# Oxygen accumulation in photobioreactors

Cláudia Sousa

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Cláudia Alexandra da Fonseca e Sousa

**Thesis committee**

**Promotor**
Prof. dr. ir. R. H. Wijffels
Professor at Bioprocess Engineering
Wageningen University

**Co-promotor**
Dr. ir. M.H. Vermuë
Assistant professor at Bioprocess Engineering
Wageningen University

**Other members**
Prof. dr. ir. G. Zeeman, Wageningen University, The Netherlands
Prof. dr. G.J.W. Euverink, University of Groningen, The Netherlands
Prof. dr. E. Molina Grima, University of Almeria, Spain
Dr. ir. A.J.B. van Boxtel, Wageningen University, The Netherlands

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# Oxygen accumulation in photobioreactors

**Cláudia Alexandra da Fonseca e Sousa**

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# Chapter 1. GENERAL INTRODUCTION & THESIS OUTLINE

## 1.1. Microalgae

Phototrophic microalgae are prokaryotic or eukaryotic microscopic organisms that are able to utilize light energy and use it to incorporate inorganic carbon in the form of dissolved carbon dioxide ($CO_2$) and bicarbonate ($HCO_3^-$) into their biomass, while producing $O_2$. Along with the photoautotrophic species there are some microalgae that are capable of utilizing organic carbon from compounds such as glucose and glycerol via respiration (heterotrophs). The enormous variety in algae metabolism makes microalgae very interesting from a biotechnological point of view as they can be used for food, feed, healthcare constituents, chemicals, energy and water treatment application.

Nowadays, a lot of research is done on large-scale microalgal production using phototropic algae as they are regarded as the most promising feedstock for sustainable biodiesel production, as they can use natural sunlight as light source and are able to utilize $CO_2$ from flue gases and nutrients (P, N) from waste streams (Boelee et al., 2011; Vunjak-Novakovic et al., 2005) (Figure 1). Compared with conventional terrestrial plants that are currently used for biodiesel production, microalgae show high photosynthetic conversion efficiencies and higher areal productivities (Mata et al., 2010). They can prosper in different ecosystems and do not compete for land with crops, neither with the food market (Zeng et al., 2011).

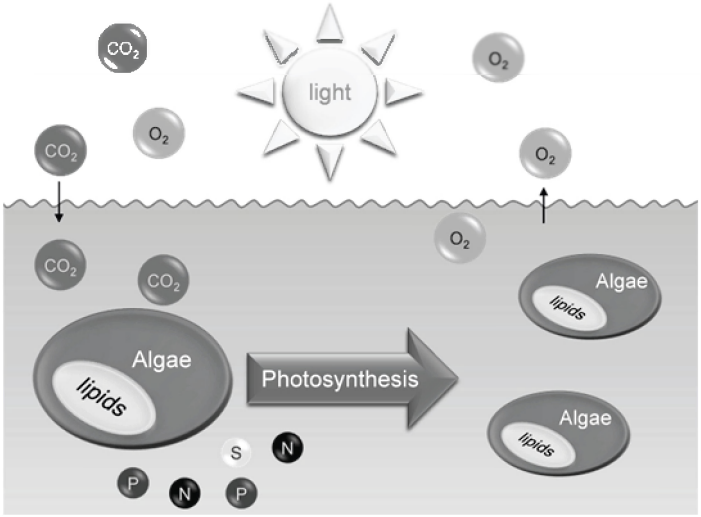



***Figure 1*** *- Schematic representation of the environmental factors influencing microalgae growth*

*Neochloris oleoabundans* is mentioned as one of the most promising strains of oleaginous green algae (Chlorophyceae) for the production of biofuels (Li et al., 2008b). It is a freshwater microalga that can also thrive at saline medium conditions. *Neochloris oleoabundans* is able to accumulate lipids in the form of triacylglycerols (also called triglyceride or TAG) and particularly at nitrogen deficient conditions, lipid contents up to 56.0 % of dry weight of biomass were reported with lipid productivities up to 133 $mg.L^{-1}.d^{-1}$, (Gouveia et al., 2009; Gouveia & Oliveira, 2009; Li et al., 2008a; Pruvost et al., 2009; Pruvost et al., 2011).

## 1.2. Large-scale outdoor cultivation

To make biofuels from microalgae a viable substitute for fossil fuels, the production should be sustainable, which means that the production should have a low environmental footprint, is economically competitive and the algal feedstock should be available in sufficient amounts to have an important impact in the energy supply chain (Amaro et al., 2012). Although the production of biofuels from microalgae is technologically feasible, the outdoor large-scale production still presents issues concerning the economic feasibility and the energy required

(Cheng & Timilsina, 2011; Norsker et al., 2011; Stephens et al., 2010a; Stephens et al., 2010b).

For the outdoor production there are two main algae cultivation systems (open and closed systems) currently being used. Open raceway ponds are the most worldwide used systems as these systems are relatively cheap. However, low biomass densities are achieved and the areal productivities and yields are relatively low (Norsker et al., 2011) and there is a high risk for contamination by bacteria and sudden collapse of the culture caused by protozoa and other predators (Carvalho et al., 2006; Pulz, 2001; Richmond, 1992). With closed systems (PBR) higher biomass densities can be achieved and there is smaller risk for contamination but there are other bottlenecks to overcome to make large-scale production in these photobioreactor systems economically feasible. The main bottleneck in closed PBRs is the high energy input that is required for mixing to provide the algae with sufficient light and carbon dioxide and other nutrients and to remove the oxygen that is produced during photosynthesis (Dismukes et al., 2008; Norsker et al., 2011; Wijffels et al., 2010). This oxygen needs to be removed to prevent adverse effects via photorespiration and photoinhibition on growth and productivity of the algae.

## 1.3. Photosynthesis, photorespiration & photoinhibition

In the photosynthetic process, water is split into oxygen and electrons with light as the driving force. The electrons are used to fix and reduce carbon dioxide to the level of sugar (triose) in the Calvin cycle from which new biomass is formed. In this Calvin cycle the enzyme Rubisco is involved in the fixation of $CO_2$. Analogous to any photosynthetic plant cell, microalgae generate oxygen during the photosynthesis (Figure 2).

In closed photobioreactors, photosynthesis causes the evolution of dissolved oxygen levels equivalent to many times the air saturation. The accumulation of photosynthetically produced $O_2$, and consequently, the high partial $O_2$ pressures results in inhibition of photosynthesis, due to photorespiration and

photoinhibition (Becker, 1994). This makes dissolved oxygen a crucial parameter to control in the microalgae production systems (Aiba, 1982).

**Photosynthesis**

Photosynthesis can be subdivided into two types of reactions: dark reactions, which are not directly influenced by light and reactions directly influenced by light. The chloroplast of a green alga contains thylakoid membranes in which two types of photosystems (PS I and PS II) are fixed and connected by an electron transport chain. In the photosynthesis, the only driving force comes from the light excitation of the special electron carriers fixed in the thylakoid membrane aided by particular protein complexes. The electrons flow through the electron transport system, from Photosystem II to Photosystem I and reduce the low energy $NADP^+$ to high energy NADPH, and transform the light energy into ATP molecules (Vacha, 1995). The latter occurs via electron transport associated with pumping of protons across the thylakoid membrane, which develops a gradient of pH that is used to the production of ATP by ATP synthase. Next, the energy and reducing power of NADPH and ATP is utilized to fix $CO_2$ via the action of Rubisco in the Calvin Cycle, or to the synthesis of saccharides in the carbon metabolism, from which new biomass is formed. ADP, Pi and $NADP^+$ are released and enter again in photosynthesis (Janssen, 2002; Vacha, 1995).

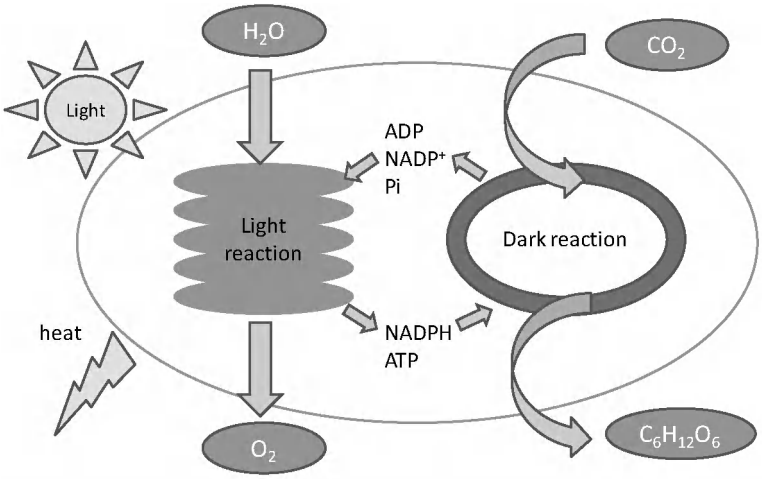



***Figure 2*** *- Simplified scheme of photosynthesis. Adapted from Bosma (2010)*

Photorespiration is a process characterized by the oxygenase activity of Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), the principal enzyme involved in the $CO_2$ assimilation in the Calvin Cycle (Figure 2) (Raven & Larkum, 2007; Vacha, 1995). $O_2$ inhibits $CO_2$ fixation *in vivo* through competitive inhibition of the Rubisco. The oxygenase and carboxylase selectivity of Rubisco is closely related to

the $CO_2/O_2$ ratio in its vicinity. This ratio decreases in conditions of high levels of oxygen, leading to the reduction of the carboxylase activity and the increase of the oxygenase activity (Osmond, 1981; Raso et al., 2011). The enhanced oxygenase activity results in a decrease of the microalgae productivity. To overcome the oxygenase/carboxylase duality of Rubisco, and its low affinity for $CO_2$, many algae acquired $CO_2$-concentrating mechanisms (CCM), which are evolutionary mechanisms that use energy to increase $CO_2$ concentrations in the proximity of Rubisco (Giordano et al., 2005).

**Photorespiration**

When Rubisco fixes $CO_2$ without photorespiration per one molecule Ribulose 1,5 biphosphate (RuBP) two molecules of 1,3-biphosphateglycerate (1,3bPGA) are formed. One of the 1,3bPGA is used to generate ATP in the Calvin cycle, while the other can be used as building block for sugars to be used to form biomass components. On the other hand, if $O_2$ is fixed, only one molecule of 3-phosphoglycerate is formed and one molecule of Glycolate 2-phosphate (G2P). G2P is converted in glycoxylate, and finally in 1,3bPGA, at the cost of $CO_2$ and ammonia ($NH_4^+$). All these processes require ATP and NADPH, which are generated in the light reaction of photosynthesis. Consequently, when photorespiration occurs, less energy is available for microalgal growth, decreasing the yield of microalgal biomass on light energy (Kliphuis et al., 2010).

Photoinhibition is another mechanism which evokes algal growth inhibition, but this process only happens at high light conditions. At these high light conditions an excessive amount of electrons is generated at Photosystem II and these electrons react with photosynthetically produced oxygen, forming oxygen radicals (Figure 3) (Murata et al., 2007).

**Water-water cycle**

In the water-water cycle the photoreduction of oxygen to water takes place in PS I by the electrons generated in PS II (Asada, 1999). In the water-water cycle, the electrons are used again to reduce oxygen, via the reactive oxygen species superoxide and hydrogen peroxide, with the help of the enzymes, superoxide dismutase (CuZn-SOD) and peroxidase (APX). These reduced and reactive oxygen species are converted to water, hence the name water-water cycle. This cycle is also known as the Mehler reaction or pseudo-cyclic electron transport and serves to scavenge the reactive oxygen radials and toxic $H_2O_2$. This process occurs only at high (i.e over-saturating) light intensities and/or nutrient limitations, leading to an over-reduction of the photosynthetic system (Asada, 1999; Ledford & Niyogi, 2005).

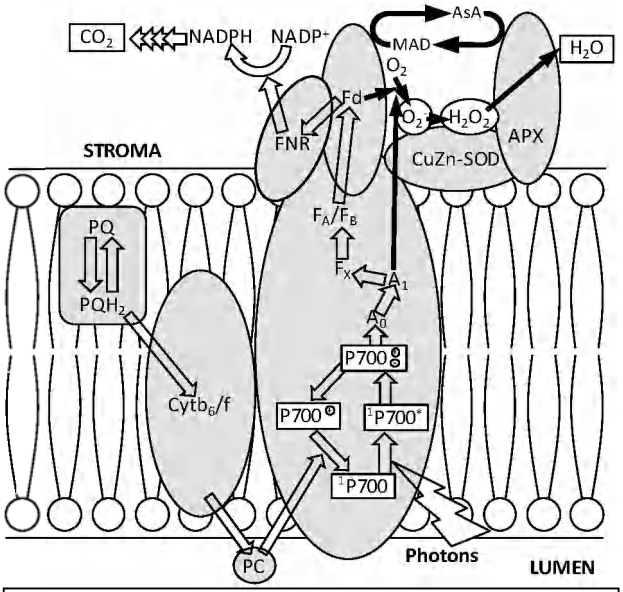
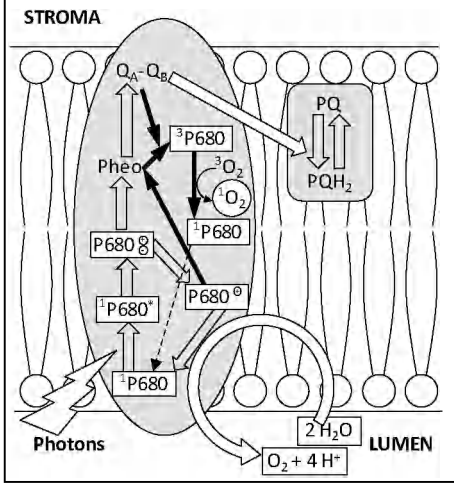



***Figure 3*** *- Formation of oxygen radicals in the water-water cycle during photosynthesis. Adapted from Asada (2006)*

These oxygen radicals are usually dealt with in the water-water cycle, but when too many electrons are generated, the enzymes in the water-water cycle are no longer capable of dealing with the surplus of electrons and oxygen radicals and other reactive oxygen species (ROS) such as $H_2O_2$ are accumulating and this has a destructive effect on biological systems (Asada, 2006; Asada, 1999; Endo & Asada, 2008).

**Photoactivation**




Photons captured by P680 cause excitation of P680 and when it falls back to the ground state the energy is used to reduce water resulting in formation of oxygen and $4H^+$ ions. In case of high light conditions a surplus of light falls at P680 and the surplus of energy is transferred to other pigments (Pheo, $Q_A$-$Q_B$) and used for formation of triplet state P680 ($^3$P680). The energy released when $^3$P680 returns to the ground singlet state ($^1$P680) the energy is partly used to transfer triplet state oxygen ($^3O_2$) into the highly reactive singlet oxygen ($^1O_2$).

***Figure 4*** *- Generation of singlet oxygen. Adapted from Asada (2006)*

Furthermore, the formation of singlet oxygen is bound to occur at high light intensities. This highly reactive compound is produced photochemically, via photoactivation (Triantaphylides et al., 2008) (Figure 4). The singlet oxygen can "attack" photosynthetic pigments in PSII, causing photo-oxidative damage.

Although the inhibiting effects of oxygen have been described in detail, in only a few studies the effect of $O_2$ was measured as parameter, independent of the light (Kliphuis et al., 2011; Molina et al., 2001; Ogren, 1984; Raso et al., 2011). The effects on growth thus often reflect a combined effect of light, pH and oxygen on photosynthesis (Torzillo et al., 1998; Ugwu et al., 2007).

## 1.4. Thesis outline

In this thesis the effect of accumulating oxygen on the growth of *Neochloris oleoabundans* is studied at sub- and near-saturating light conditions in a fully controlled photobioreactor operated in turbidostat mode (Figure 5) to reveal to what extent the oxygen inhibits the growth of the algae and what oxygen concentrations would still lead to acceptable growth rates of the algae. In addition, the oxygen accumulation which may occur in closed photobioreactors was mimicked and the effects of these dynamically changing oxygen conditions on the algal growth were examined. With the generated knowledge it has been possible to predict the minimum amount of energy needed to keep the oxygen

level sufficiently low and calculate the energy savings that are possible when an out-door tubular photobioreactor system is operated at large scale using these minimized mixing and degassing conditions.


***Figure 5*** *- Lab-scale CSTR photobioreactor used in the experiments.*

Chapter 2 of this thesis describes the effect of partial oxygen pressure on growth of *Neochloris oleoabundans* at sub-saturating light intensity in a fully-controlled stirred tank photobioreactor. In this work we studied 3 different partial oxygen pressures ($P_{O_2}$) and evaluate its effect on specific growth rates. 2 different partial carbon dioxide pressures ($P_{CO_2}$) at the highest $P_{O_2}$=0.84 bar were considered and used to confirm the presence of photorespiration phenomena and to overcome it. In chapter 3 a continuation of the study of chapter 2 was performed at near-saturating light intensities. The effect of partial oxygen pressure on growth of *Neochloris oleoabundans* was evaluated at 4 different partial oxygen pressures ($P_{O_2}$= 0.24; 0.42; 0.63; 0.84 bar) as well as an increase of the $P_{CO_2}$ from 0.007 to 0.02 bar at $P_{O_2}$ of 0.84. The specific growth rates and pigment content of the microalgae were used to assess the presence of phenomena like photoaclimation, photoinhibition and photooxidative damage.

In chapter 4 and 5 the effects of the increase of the oxygen concentration followed by a decrease of the oxygen in the degasser were simulated at low and

high light intensity and the effect of a 10 times elongation of the residence time at in the solar receiver was investigated. The light regimes used were: continuous light ON; 30 minutes of light ON followed by 6 minutes lights OFF and 300 minutes of light ON followed by 6 minutes lights OFF. The effect of dynamically changing oxygen concentrations from $P_{O_2}$=0.21 bar to $P_{O_2}$=0.63 bar followed by subsequent degassing to $P_{O_2}$=0.21 bar during the dark period resulted in similar specific growth rates. The decrease of the algae specific growth observed when applying different light regimes, shows that the exposure of the algae cells to dark periods in the degasser has bigger negative impact than the temporary exposure to accumulating oxygen concentrations in the solar receiver. In chapter 5 these results the same results were found indicating that the algae do not experience the expected photo-oxidative inhibition caused by high oxygen concentration in combination with high light, as long as the oxygen is removed via regular degassing.

In chapter 6 a model which was developed to calculate the energy and costs associated to microalgae biomass production in the Netherlands for three different systems at 100 ha scale was used to evaluate the implementation of the findings described on the previous chapters. Chapter 6 is a general discussion about the effect of the reduction of the costs for degassing and its influence on the overall costs and net energy balance.

## 1.5. References

Aiba, S. 1982. Growth kinetics of photosynthetic microorganisms. Advances in Biochemical Engineering, 23, 85-156.

Amaro, H.M., Macedo, A.C., Malcata, F.X. 2012. Microalgae: An alternative as sustainable source of biofuels? Energy, 44(1), 158-166.

Asada, K. 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiology, 141(2), 391-396.

Asada, K. 1999. The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. Annual Review of Plant Physiology and Plant Molecular Biology, 50(1), 601-639.

Becker, E.W. 1994. Microalgae: biotechnology and microbiology. Cambridge University Press, Cambridge.

Boelee, N.C., Temmink, H., Janssen, M., Buisman, C.J.N., Wijffels, R.H. 2011. Nitrogen and phosphorus removal from municipal wastewater effluent using microalgal biofilms. Water Research, 45(18), 5925-5933.

Bosma, R. 2010. Towards high productivities of microalgae in photobioreactors, PhD thesis, University of Wageningen, The Netherlands.

Carvalho, A.P., Meireles, L.A., Malcata, F.X. 2006. Microalgal reactors: A review of enclosed system designs and performances. Biotechnology Progress, 22(6), 1490-1506.

Cheng, J.J., Timilsina, G.R. 2011. Status and barriers of advanced biofuel technologies: A review. Renewable Energy, 36(12), 3541-3549.

Dismukes, G.C., Carrieri, D., Bennette, N., Ananyev, G.M., Posewitz, M.C. 2008. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Current Opinion in Biotechnology, 19(3), 235-240.

Endo, T., Asada, K. 2008. Photosystem I and photoprotection: cyclic electron flow and water-water cycle. in: Photoprotection, Photoinhibition, Gene Regulation, and Environment, (Eds.) B. Demmig-Adams, W.W. Adams, A.K. Mattoo, Vol. 21, Springer. Dordrecht, pp. 205-221.

Giordano, M., Beardall, J., Raven, J.A. 2005. $CO_2$ Concentrating Mechanisms in Algae: Mechanisms, Environmental Modulation, and Evolution. Annual Review of Plant Biology, 56, 99-131.

Gouveia, L., Marques, A.E., da Silva, T.L., Reis, A. 2009. *Neochloris oleabundans* UTEX #1185: a suitable renewable lipid source for biofuel production. Journal of Industrial Microbiology and Biotechnology, 1-6.

Gouveia, L., Oliveira, A.C. 2009. Microalgae as a raw material for biofuels production. Journal of Industrial Microbiology & Biotechnology, 36(2), 269-274.

Janssen, M. 2002. Cultivation of microalgae: effect of light/dark cycles on biomass yield, PhD thesis, University of Wageningen, The Netherlands.

Kliphuis, A.M.J., de Winter, L., Vejrazka, C., Martens, D.E., Janssen, M., Wijffels, R.H. 2010. Photosynthetic efficiency of *Chlorella sorokiniana* in a turbulently mixed short light-path photobioreactor. Biotechnology Progress, 26(3), 687-696.

Kliphuis, A.M.J., Martens, D.E., Janssen, M., Wijffels, R.H. 2011. Effect of $O_2$:$CO_2$ ratio on the primary metabolism of *Chlamydomonas reinhardtii*. Biotechnology and Bioengineering, 108(10), 2390-2402.

Ledford, H.K., Niyogi, K.K. 2005. Singlet oxygen and photo-oxidative stress management in plants and algae. Plant Cell and Environment, 28(8), 1037-1045.

Li, Y., Horsman, M., Wang, B., Wu, N., Lan, C.Q. 2008a. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Applied Microbiology and Biotechnology, 81(4), 629-636.

Li, Y., Horsman, M., Wu, N., Lan, C.Q., Dubois-Calero, N. 2008b. Biofuels from Microalgae. Biotechnology Progress.

Mata, T.M., Martins, A.A., Caetano, N.S. 2010. Microalgae for biodiesel production and other applications: A review. Renewable and Sustainable Energy Reviews, 14(1), 217-232.

Molina, E., Fernandez, F.G.A., Chisti, M.Y. 2001. Tubular photobioreactor design for algal cultures. Journal of Biotechnology, 92, 113-131.

Murata, N., Takahashi, S., Nishiyama, Y., Allakhverdiev, S.I. 2007. Photoinhibition of photosystem II under environmental stress. Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1767(6), 414-421.

Norsker, N.-H., Barbosa, M.J., Vermuë, M.H., Wijffels, R.H. 2011. Microalgal production -- A close look at the economics. Biotechnology Advances, 29(1), 24-27.

Ogren, W.L. 1984. Photorespiration: Pathways, regulation, and modification. Annual Review of Plant Physiology, 35(1), 415-442.

Osmond, C.B. 1981. Photorespiration and photoinhibition. Some implications for the energetics of photosynthesis. Biochimica et Biophysica Acta, 639, 77-98.

Pruvost, J., Van Vooren, G., Cogne, G., Legrand, J. 2009. Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. Bioresource Technology, 100(23), 5988-5995.

Pruvost, J., Van Vooren, G., Le Gouic, B., Couzinet-Mossion, A., Legrand, J. 2011. Systematic investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel application. Bioresource Technology, 102, 150-158.

Pulz, O. 2001. Photobioreactors: production systems for phototrophic microorganisms. Applied Microbiology and Biotechnology, 57, 287-293.

Raso, S., van Genugten, B., Vermuë, M., Wijffels, R.H. 2011. Effect of oxygen concentration on the growth of *Nannochloropsis sp.* at low light intensity. Journal of Applied Phycology, 1-9.

Raven, J.A., Larkum, A.W.D. 2007. Are there ecological implications for the proposed energetic restrictions on photosynthetic oxygen evolution at high oxygen concentrations? Photosynthesis Research, 94, 31-42.

Richmond, A. 1992. Open systems for the mass production of photoautotrophic microalgae outdoors: physiological principles. Journal of Applied Phycology, 4(3), 281-286.

Stephens, E., Ross, I.L., King, Z., Mussgnug, J.H., Kruse, O., Posten, C., Borowitzka, M.A., Hankamer, B. 2010a. An economic and technical evaluation of microalgal biofuels. Nature Biotechnology, 28(2), 126-128.

Stephens, E., Ross, I.L., Mussgnug, J.H., Wagner, L.D., Borowitzka, M.A., Posten, C., Kruse, O., Hankamer, B. 2010b. Future prospects of microalgal biofuel production systems. Trends in Plant Science, 15(10), 554-564.

Torzillo, G., Bernardini, P., Masojidek, J. 1998. On-line monitoring of chlorophyll fluorescence to assess the extent of photoinhibition of photosynthesis induced by high oxygen concentration and low temperature and its effect on the productivity of outdoor cultures of *Spirulina platensis* (cyanobacteria). Journal of Phycology, 34 504 - 510

Triantaphylides, C., Krischke, M., Hoeberichts, F.A., Ksas, B., Gresser, G., Havaux, M., Van Breusegem, F., Mueller, M.J. 2008. Singlet oxygen is the major reactive

oxygen species involved in photooxidative damage to plants. Plant Physiology, 148(2), 960-968.

Ugwu, C.U., Aoyagi, H., Uchiyama, H. 2007. Influence of irradiance, dissolved oxygen concentration, and temperature on the growth of *Chlorella sorokiniana*. Photosynthetica, 45(2), 309-311.

Vacha, F. 1995. The role of oxygen in photosynthesis. Photosynthetica, 31(3), 321-334.

Vunjak-Novakovic, G., Kim, Y., Wu, X.X., Berzin, I., Merchuk, J.C. 2005. Air-lift bioreactors for algal growth on flue gas: Mathematical modeling and pilot-plant studies. Industrial & Engineering Chemistry Research, 44(16), 6154-6163.

Wijffels, R.H., Barbosa, M.J., Eppink, M.H.M. 2010. Microalgae for the production of bulk chemicals and biofuels. Biofuels, Bioproducts and Biorefining, 4(3), 287-295.

Zeng, X.H., Danquah, M.K., Chen, X.D., Lu, Y.H. 2011. Microalgae bioengineering: From $CO_2$ fixation to biofuel production. Renewable & Sustainable Energy Reviews, 15(6), 3252-3260.

# Chapter 2. GROWTH OF THE MICROALGAE *NEOCHLORIS OLEOABUNDANS* AT HIGH PARTIAL OXYGEN PRESSURES AND SUB-SATURATING LIGHT INTENSITY

Claudia Sousa[1, 2], Lenneke de Winter [1, 2], Marcel Janssen[2], Marian H. Vermuë[2] and Rene H. Wijffels[2]

[1.] Wetsus, P.O. Box 1113, 8900 CC Leeuwarden, The Netherlands

[2.] Bioprocess Engineering, Wageningen University, P.O. Box 8129, 6700 EV, Wageningen, The Netherlands

**Abstract -** The effect of partial oxygen pressure on growth of *Neochloris oleoabundans* was studied at sub-saturating light intensity in a fully-controlled stirred tank photobioreactor. At the three partial oxygen pressures tested ($P_{O_2}$= 0.24; 0.63; 0.84 bar), the specific growth rate was 1.38; 1.36 and 1.06 $day^{-1}$, respectively. An increase of the $P_{CO_2}$ from 0.007 to 0.02 bar at $P_{O_2}$ of 0.84 bar resulted in an increase in the growth rate from 1.06 to 1.36 $day^{-1}$. These results confirm that the reduction of algal growth at high oxygen concentrations at sub-saturating light conditions is mainly caused by competitive inhibition of Rubisco. This negative effect on growth can be overcome by restoring the $O_2/CO_2$ ratio by an increase in the partial carbon dioxide pressure. In comparison to general practice ($P_{O_2}$=0.42 bar), working at partial $O_2$ pressure of 0.84 bar could reduce the energy requirement for degassing by a factor of 3 to 4.

**Key words:** *Neochloris oleoabundans*, photosynthesis, oxygen inhibition, photorespiration, photobioreactor

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## 2.1. Introduction

Lipid-rich photoautotrophic microalgae such as *Neochloris oleoabundans* are promising renewable resources for biodiesel production, because of their high productivity and because their production does not have to compete with food (Chisti, 2007; Schenk et al., 2008; Wijffels and Barbosa, 2010). However, large-scale outdoor production of microalgae is not yet economically feasible. High energy inputs are required for mixing to provide the algae with light and carbon dioxide and to remove the photosynthetically produced oxygen (Dismukes et al., 2008; Norsker et al., 2010; Wijffels et al., 2010). When oxygen accumulates in the culture medium, photoinhibition and photorespiration take place, leading to a decrease in biomass yield on light energy (Torzillo et al., 1998). Photoinhibition occurs mainly at high and over-saturating light intensities. At those conditions an excess of electrons is generated in Photosystem II and these will react with the photosynthetically produced oxygen, leading to the formation of oxygen radicals (Murata et al., 2007, Pospíšil, 2011). In addition, light stimulates the formation of singlet oxygen via photo-activation (Triantaphylides et al., 2008) which damages the photosystems of the algal cells.

Photorespiration is associated with the oxygenase activity of the enzyme Rubisco. The accumulation of oxygen ($O_2$) will lead to an increase of the local $O_2/CO_2$ ratio and, consequently, to reduced carboxylase activity and increased oxygenase activity. Overall the productivity of the microalgae culture will decrease (Osmond, 1981). Photorespiration only occurs in the dark reaction of photosynthesis and is thus not related to formation of reactive oxygen species occurring at high- and over-saturating light conditions. At sub-saturating light intensities photoinhibition is negligible small, which makes photorespiration the dominant process leading to reduced photosynthetic yield under oxygen accumulation.

During photorespiration, $CO_2$ and ammonium ($NH_4^+$) are lost and their re-fixation requires additional ATP and NADPH. This means that less energy is available for growth and the biomass yield on light energy will decrease when photorespiration occurs (Kliphuis et al., 2010). The photorespiratory pathway thus has an influence on the photosynthetic yield which can be defined as the amount of $CO_2$ fixed per amount of light energy absorbed and, as such, will directly influence the productivity of microalgae cultures.

Although the inhibiting effects of oxygen have been described in detail, in hardly any of the reported studies the effect of $O_2$ was measured as independent parameter (Kliphuis et al., 2011; Molina et al., 2001; Ogren, 1984; Raso et al., 2011). The effects on growth often reflect a combined effect of light, pH and oxygen on photosynthesis (Torzillo et al., 1998; Ugwu et al., 2007). In the present study, the effect of partial oxygen pressures on the growth of *Neochloris oleoabundans* at sub-saturating light conditions in a fully controlled photobioreactor operated in turbidostat mode was determined. The specific growth rate as well as the biomass yield on photons under three different partial oxygen pressures ($P_{O_2}$ = 0.24; 0.63; 0.84 bar) were measured. At the highest partial oxygen pressure (0.84 bar) the effect of increasing the partial carbon dioxide pressure was determined to assess whether photorespiration could be reduced by decreasing the $O_2/CO_2$ ratio in the microalgae culture.

## 2.2. Material and methods

### 2.2.1. Cultures and medium

*Neochloris oleoabundans* (UTEX 1185) cultures were maintained in 100 ml liquid cultures in 250 ml Erlenmeyer flasks closed with porous stoppers (Bio-silico, Hirschmann Laborgeräte GmbH & Co.KG, Germany). The flasks were placed in an incubator with orbital shaker (Innova 44R, New Brunswick Scientific, USA) under fluorescent light (40 µmol $m^{-2}$ $s^{-1}$) at 25 °C and 120 rpm. The air inside the incubator was enriched with 2% carbon dioxide.

Adapted f/2 medium (Guillard and Ryther, 1962) was used to grow and maintain *Neochloris oleoabundans* cultures. The medium was composed of artificial sea water (in mM): NaCl, 419; $MgCl_2.6H_2O$, 48.2; $CaCl_2.2H2O$, 3.6; $Na_2SO_4$, 22.5; $K_2SO_4$, 4.9. The artificial sea water was enriched with the following nutrients (in mM): $NaH_2PO_4.2H_2O$, 2.50; $NaNO_3$, 32; trace elements (in µM): EDTA-FeNa, 29.3 $CuSO_4.5H_2O$, 0.10; $Na_2MoO_4.2H_2O$, 0.07; $ZnSO_4.7H_2O$, 0.19; $CoCl_2.6H_2O$, 0.19; $MnCl_2.4H_2O$, 2.27; vitamins (µg $L^{-1}$): thiamine, 200; biotine, 1.00; cyanocobalamine, 1.00. The pH was adjusted to 7.8 with 0.5 M NaOH and the medium was sterilized via filtration through 0.22 µm filters. For the reactor

experiments the culture media was enriched with $NaHCO_3$ to a final concentration of 10 mM.

### 2.2.2. Photobioreactor

Continuous turbidostat experiments were performed in a 3 L jacketed bioreactor (Applikon Biotechnology, The Netherlands) (Fig. 1). The internal diameter was 12.5 cm and the liquid volume was 2 L resulting in an illuminated surface of 0.061 $m^2$ (Ar). The reactor was equipped with a marine impeller. All sensors and regulators were connected to an Ez-controller equipped with Bioexpert© software (Applikon Biotechnology, The Netherlands).

The measured and controlled process parameters were: pH; temperature; oxygen and carbon dioxide partial pressure in the liquid phase ($P_{O_2}$ and $P_{CO_2}$); liquid level; stirrer speed and optical density (OD). The pH was maintained in the range 7.8 ± 0.15 by automatic addition of gaseous carbon dioxide ($CO_2$). Temperature was maintained at 25°C. The partial oxygen pressure increased as a result of photosynthesis and was maintained at the desired level by automatic addition of gaseous dinitrogen ($N_2$). The speed of the marine impeller was kept constant at 250 rpm. The optical density was controlled at 0.55 CU using a turbidity sensor (ASD19-N, Optek, Germany) connected to a peristaltic pump automatically adding fresh medium to the reactor when required. The liquid level was maintained by a level sensor controlling another peristaltic pump to remove excess culture.

The Clark type $P_{O_2}$ sensor (LowDrift sensor, Applisens, The Netherlands) was calibrated inside the photobioreactor with growth medium using pure $O_2$ giving a $P_{O_2}$ of 1 bar. The $P_{CO_2}$ sensor was based on a pH sensor equipped with a $CO_2$ selective membrane (InPro 5000, Mettler Toledo, Switzerland) and was calibrated similarly but with 4% v/v $CO_2$ enriched air giving a $P_{CO_2}$ of 0.04 bar.

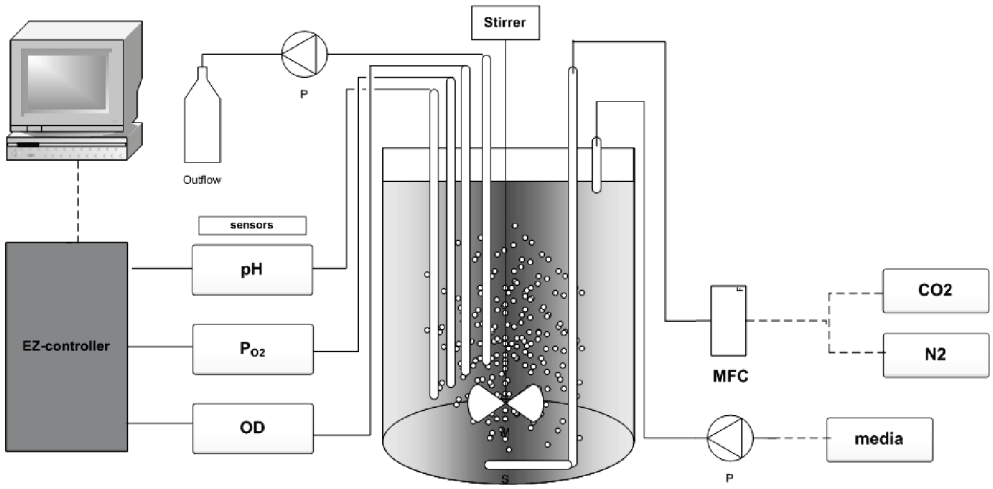


***Figure 1*** *- Experimental set-up (not on scale).*
*S = gas distributor, M = marine impeller, P = peristaltic pumps, $P_{O_2}$ = partial oxygen pressure, OD = optical density or turbidity, Ez- controller = reactor control unit, MFC = mass flow controllers for carbon dioxide ($CO_2$) and nitrogen ($N_2$)*

The culture was continuously illuminated with two light panels (20×20 cm) with red (627 nm) LED lights (SL3500, Photon Systems Instruments, Czech Republic). Under operating conditions the emission peak shifts to higher wavelengths due to lamp heating and a peak intensity of 635 nm was used in the calculation of the light gradient. The panels were placed at both sides of the reactor and a plate of opal glass was placed in front of the light panels to ensure better light distribution. Reflective material was placed on the screens surrounding the reactor to homogenize the light further (Appendix 2A). The incident light intensity was measured with a PAR quantum sensor (model SA-190, LiCor Biosciences, USA) before the start of each experimental run. The measurements were done at different heights and radial positions to determine the average incident photon flux density (PFD avg). The average value for the different experiments was always between 187 and 210 µmol $m^{-2}$ $s^{-1}$. (Appendix 2A)

## 2.2.3. Dry weight concentration

The determination of the dry weight concentration of reactor samples was done in triplicate. Samples of 5 mL were diluted with 10 ml ammonium formate (0.5 M).

The diluted samples were filtered over pre-weighed glass fiber-filters (Whatman GF/F) and washed with an additional 40 ml of ammonium formate (0.5 M). The filters were dried at 95 °C for 24 hours in aluminum trays, allowed to cool down in a desiccator for at least two hours, and weighed (ME235P-SD, Sartorius, Germany).

## 2.2.4. Specific absorption coefficient

Light absorption by the microalgae cells was measured in a specialized spectrophotometer set-up to minimize the effect of light scattering on the absorption measurement. A sample with extinction at 680 nm (chlorophyll absorption peak) between 1.8 and 2.2 was used, as measured in a 1 cm cuvette in a spectrophotometer (Beckman DU®640, Beckman Coulter, USA). The absorbance was then measured with a fiber optic CCD based spectrophotometer (Avantes, The Netherlands). The sample was placed in 2 mm light path cuvette (Hellma, 100.099-OS, 2 mm light path) and illuminated with an AvaLight-Hal light source via a FC-IR600-1-M fiber equipped with a collimating lens. An integrating sphere (AvaSphere-50) was directly placed behind the cuvette and connected to the Avantes Avaspec-2048 detector via another FC-IR600-1-M fiber. The resulting absorbance was measured from 400 nm to 750 nm. The average absorbance from 740 nm - 750 nm was subtracted from the absorbance between 400 nm and 700 nm, thus correcting for residual scattering (Dubinsky et al., 1986). The wavelength-dependent dry weight specific absorption coefficient ($a_\lambda$ $m^2$ $g^{-1}$) was calculated based on the absorbance (*ABS*) at wavelength $\lambda$, the dry weight ($C_x$, g $m^{-3}$), the light path of the cuvette ($l$, m) (equation 1):

$$a_\lambda = \frac{2.303 \cdot ABS_\lambda}{C_x \cdot l} \quad \left[m^2 \cdot g^{-1}\right] \tag{1}$$

The light gradient inside the bioreactor has been estimated using Beers' law and the geometrical relationship derived for cylindrical vessels (Evers, 1991) with a modification to account for the use of a flat cosine receiver as light sensor (Appendix 2B.). The biomass-specific absorption coefficient was used for this calculation. The full absorption spectrum of a diluted *Neochloris oleoabundans* culture grown at 200 µmol $m^{-2}$ $s^{-1}$ can be found in Appendix 2C.

### 2.2.5. Photosynthesis-irradiance curve (PI- curve)

In order to confirm that the (average) PFD inside the photobioreactor indeed imposes sub-saturating light conditions, a photosynthesis irradiance (PI) curve was determined for *Neochloris oleoabundans*. The sample for the PI curve measurement was taken from a batch culture in a flat panel photobioreactor with 2 mm light path. Light intensity on the surface was set to 200 µmol $m^{-2}$ $s^{-1}$, temperature was 30 °C, pH was 7.5 and the air flow rate was 0.70 L $L^{-1}$ $min^{-1}$ enriched with 2% $CO_2$. The sample was taken when the biomass density was 1.5 gDW $L^{-1}$ and the algae were exposed to relatively low light intensities. The specific oxygen production rate was measured with a Biological Oxygen Monitor (BOM) (Hansatech Instruments Limited, Norfolk England). The DW3 electrode chamber of Hansatech had a light path of 2 cm which was illuminated with a LH36/2R red LED light source (peak wavelength 655 nm). The electrode chamber was filled with 9 ml of buffered medium without any carbon. The medium was then flushed with pure dinitrogen for 10 minutes. Subsequently, 3 ml of sample and 80 µL of 0.75 M $NaHCO_3$ solution were added. The rate of dark respiration was followed for several minutes after which the net specific oxygen production rate was followed for 3 minutes at 10 different PFD levels. Corresponding gross rates of photosynthesis were then calculated by adding the measured dark respiration to the measured net rates of oxygen evolution.

## 2.3. Results and discussion

### 2.3.1. Light regime

Photorespiration is expected to be the dominant process leading to growth inhibition because of oxygen accumulation under sub-saturating light conditions.

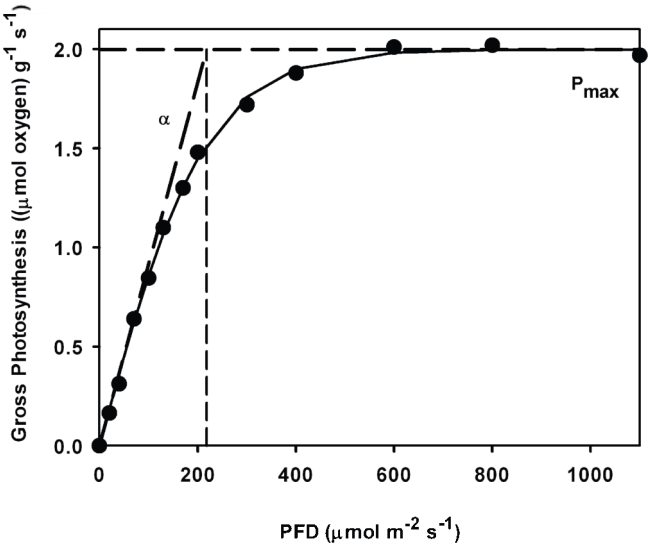


***Figure 2*** *- Gross rate of photosynthesis of Neochloris oleoabundans versus irradiance cells in µmol oxygen gDW$^{-1}$ s$^{-1}$ at different photon flux densities (PFD)*

To ensure that the average light intensity experienced by the algae inside the photobioreactor was indeed sub-saturating, photosynthesis irradiance (PI) curve was determined for *Neochloris oleoabundans*. Figure 2 shows the PI curve of *Neochloris oleoabundans* acclimated to low light conditions. At low irradiance levels, the gross photosynthetic rate of the algae is linearly proportional to the irradiance. The graph shows that the cells experienced sub-saturating light below 218 µmol m$^{-2}$ s$^{-1}$.The PI curve thus confirms that the algae grew under light limiting conditions at the photon flux density applied, which ranged from an average of 210 µmol m$^{-2}$ s$^{-1}$ at the reactor surface to values as low as 25 µmol m$^{-2}$ s$^{-1}$ in the reactor centre (Appendix B). Although the light levels at the bioreactor surface were above 200 µmol m$^{-2}$ s$^{-1}$, the algae were only shortly exposed to these saturating light levels considering the mixing of the liquid. This temporal exposure apparently did not result in significant photoinhibition considering the high growth rate measured. In addition, the high biomass yields on photons are comparable to growth rates found by Pruvost et al. (2009). Apparently the temporal exposure to high light locally at the reactor surface did not negatively affect growth.

## 2.3.2. Growth and productivity

Figure 3 shows a typical run at 0.21 bar partial oxygen pressure. The bioreactor was running batch-wise until the desired optical density of 0.55 CU was reached and then the turbidostat control was activated. The optical density (0.55 CU) corresponds to a biomass concentration of 0.40 gDW $L^{-1}$ for *Neochloris oleoabundans*. The biomass density was kept constant and the specific growth rate was determined when the cells were acclimated to the culture conditions and the dilution rate did not change for 4 consecutive days.

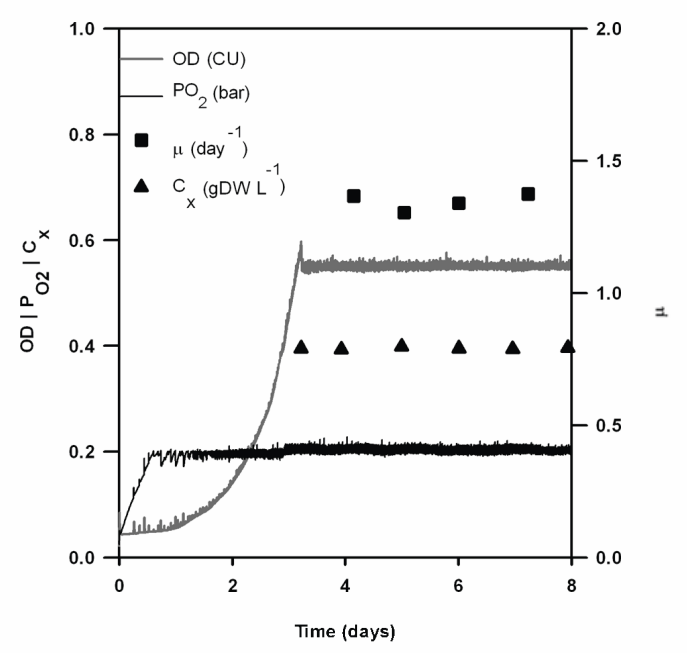


***Figure 3*** *- Graphical representation of the turbidostat experiment with Neochloris oleoabundans at an partial oxygen pressure of 0.21 bar.*
*Presented are the partial oxygen pressure ($P_{O_2}$), optical density (OD), dry weight concentration ($C_x$) and the specific growth rate ($\mu$).*

At a dissolved oxygen concentration of 100% air saturation ($P_{O_2}$ = 0.21 bar), a specific growth rate of 1.38 $day^{-1}$ was measured (Fig. 3). This corresponds to a daily volumetric biomass productivity of 0.55 gDW $L^{-1}$ $day^{-1}$ and a biomass yield on photons of 1.04 gDW mol-$ph^{-1}$. Using the daily volumetric biomass productivity for

*Neochloris oleoabundans* measured by Pruvost et al. (2009) and calculating the photon daily volumetric productivity with the provided data of incident light flux and illuminated surface to volume ratio of the reactor, a biomass yield on photons of 0.71 gDW mol-ph$^{-1}$ was calculated. This comparison shows that *Neochloris oleoabundans* shows high biomass yields grown on a marine salt water medium or on a freshwater BBM medium as used by Pruvost and coworkers. This outcome is actually not surprising since *Neochloris oleoabundans* (UTEX 1185) has been isolated from an arid soil (Guiry, 2011).

Table 1 shows the compilation of the results obtained for the different $P_{O_2}$ tested. The specific growth rate and biomass concentration were calculated. Going from air saturation levels ($P_{O_2}$= 0.21 bar) to three times air saturation ($P_{O_2}$ = 0.63 bar), the specific growth rate remained nearly the same but reaching four times air saturation ($P_{O_2}$ = 0.84 bar), a decrease in growth rate was observed. When testing *Neochloris oleoabundans* under a $P_{O_2}$ of 0.84 bar, the specific growth rate decreased from 1.36 to 1.06 day$^{-1}$.

***Table 1*** *- Specific growth rate, biomass concentration of the microalgae Neochloris oleoabundans at different oxygen and carbon dioxide partial pressures.*

| $NaHCO_3$(mM) | $P_{O_2}$ (bar) | $P_{CO_2}$ (bar) | μ (day$^{-1}$) ±Stdev | $C_x$ (g Kg$^{-1}$) ±Stdev |
|---|---|---|---|---|
| 10 | 0.21 | 0.007 | 1.38 ± 0.17 | 0.40 ± 0.002 |
| 10 | 0.63 | 0.007 | 1.36 ± 0.18 | 0.41 ±0.04 |
| 10 | 0.84 | 0.007 | 1.06 ± 0.02 | 0.39 ± 0.004 |
| 30 | 0.84 | 0.020 | 1.36 ± 0.002 | 0.39 ± 0.004 |

Molina et al. (2001) observed a decrease in photosynthetic activity of *Phaeodactylum tricornutum* at $P_{O_2}$ higher than 0.21 bar. Although a decrease in growth rate upon an increase of the partial pressure of oxygen from 0.63 to 0.84 bar was found in the current study, no decrease was observed upon changing its partial pressure from 0.21 to 0.63 bar. This result could be due to the activity of a carbon concentration mechanisms (CCM) present in *Neochloris oleoabundans*. CCMs are evolutionary mechanisms developed to overcome the low affinity of Rubisco for $CO_2$ and its oxygenase and carboxylase duality (Giordano et al., 2005;

Raven et al., 2008). However, the CCMs could not prevent a decrease in growth when going from partial oxygen pressure of 0.63 bar to 0.84 bar. Also, a CCM has a limited and requires a substantial energy input (Vance and Spalding, 2005).

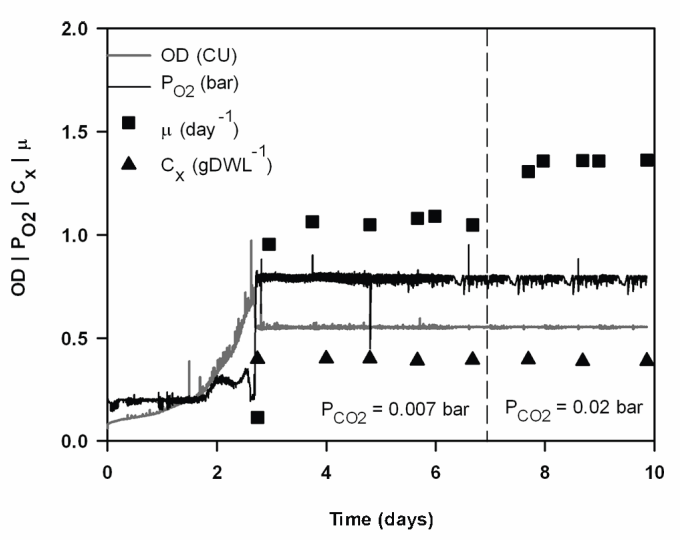


***Figure 4*** *- Graphical representation of the turbidostat experiment with Neochloris oleoabundans at a partial oxygen pressure of 0.84 bar and carbon dioxide partial pressures of 0.007 and 0.02 bar.*
*Presented are the partial oxygen pressure ($P_{O_2}$), optical density (OD), dry weight concentration (Cx) and the specific growth rate (μ).*

In order to overcome the inhibitory effect of oxygen at high partial pressures, the $CO_2$ concentration was increased by the addition of extra $NaHCO_3$ to the culture medium. The $NaHCO_3$ concentration in the medium was increased from 10 mM to 30 mM corresponding to an increase in the $P_{CO_2}$ of 0.007 ± 0.0001 to 0.020 ± 0.001 bar (measured value). The dilution rate and hence the specific growth rate, started to increase within the first day and reached a level of 1.36 day$^{-1}$ (Fig. 4), similar to the growth rates that were reached at $P_{O_2}$=0.21 bar and 0.63 bar. This result indicates that photorespiration is indeed responsible for the observed decrease in growth rate at higher oxygen concentration. The oxygenase/ carboxylase activity of the enzyme Rubisco is associated with the ratio between $O_2$ and $CO_2$ in the medium and once the bicarbonate ($NaHCO_3$) concentration and

the corresponding $CO_2$ concentration was increased, the specific growth rate of the algae increased again. Kliphuis et al. (2011) studied the effect of $O_2/CO_2$ ratio on the primary metabolism of *Chlamydomonas reinhardtii* under simulated production conditions and found a decrease in specific growth rate with increasing $O_2/CO_2$ ratios. Using a metabolic flux model, they quantified oxygenase/carboxylase reactions and the fluxes through the photorespiratory pathway and showed that the decrease found can be explained by the increased oxygenase activity. They found this effect already when increasing $P_{O_2}$ in the liquid phase from 0.11 to 0.26 bar at a $P_{CO_2}$ of 0.014 bar although they were working at over-saturating incident light of 600 µmol $m^{-2}$ $s^{-1}$. To conclude from both the presented study as well as from the study presented by Kliphuis et al. (2011), an increase of dissolved $O_2/CO_2$ ratios in photobioreactors will result in reduced productivity although the actual quantitative effect is species dependent. Especially *Neochloris oleoabundans* appears to tolerate high $P_{O_2}$ levels at sub-saturating light.

### 2.3.3. Implications on reactor design

Large-scale outdoor production of microalgae is not yet economically feasible. High energy inputs are required for mixing to remove the photosynthetically produced oxygen ($O_2$). Our results can help in reducing the energy requirement for cultivation systems where spatial dilution of light is applied. When using systems of vertical panel photobioreactors (Rodolfi et al., 2009), sunlight intensity can be reduced and distributed over a larger surface. In vertical flat panel reactors the photosynthetic efficiency is high because of the dilution of light (Cuaresma et al., 2011). At these reduced light levels in high density microalgae cultures the effect of photoinhibition will be absent and the negative effect of high oxygen concentrations on *Neochloris oleoabundans* can be overcome by working at elevated $CO_2$ concentrations.

When working with combustion gasses with 10 to 20 % v/v $CO_2$, it is a realistic scenario to maintain $CO_2$ partial pressures in the order of 0.02 bar while still being able to remove more than 80% of the $CO_2$ from the gas. The advantage of working at a higher partial $CO_2$ pressure is the fact that a higher partial $O_2$ pressure can be tolerated in the culture as shown in this study and there is a higher driving force

for oxygen transfer from the liquid to the gas phase. Consequently, the rate of gassing can be reduced.

As an example the reduction in gas flow rate, Fg ($m^3\ s^{-1}$) was calculated for a case where a flat panel photobioreactor was running at a dissolved $O_2$ concentration equivalent to a $P_{O_2}$ of 0.84 bar instead of $P_{O_2}$=0.42 bar (assumed to be current practice). In this calculation, we assumed a 1.5 m high panel reactor with a depth of 0.07 m. The oxygen transfer requirement (OTR) for such a system was calculated considering a volumetric oxygen production rate of 1.98 x $10^{-4}$ mol $m^{-3}$ $s^{-1}$. This oxygen production rate was estimated based on a quantum requirement which was calculated from the biomass yield on photons observed in this study (1.04 gDW mol-$ph^{-1}$) and a light energy input of 200 µmol $m^{-2}$ $s^{-1}$ on the vertical side of the panel (one side only).

The oxygen transfer coefficient $K_l.a$ ($s^{-1}$) needed to remove the oxygen produced from the photobioreactor while maintaining a dissolved oxygen concentration in the liquid phase, $C_{ol}$, of 0.537 mol $m^{-3}$ (equivalent to $P_{O_2}$=0.42 bar) or 1.073 mol $m^{-3}$ (equivalent to $P_{O_2}$=0.84 bar). The concentration of $O_2$ in the liquid at the gas-liquid interface was assumed to be in equilibrium with the oxygen level in air, so $C^*_{ol}$= 0.268 mol $m^{-3}$. The difference between $C_{ol}$ and $C^*_{ol}$ represents the driving force of $O_2$ transfer over the liquid boundary layer surrounding the gas bubbles (equation 2).

$$OTR = K_l.a \cdot \left(C_{ol} - C^*_{ol}\right) \tag{2}$$

The gas flow rate, $F_g$ ($m^3\ s^{-1}$) was calculated using the relation for $K_l$.a determined by Sierra et al., (2008) for flat panel reactors (equation 3).Where ρ (Kg $m^{-3}$) stands for the density of the liquid; g ($m^{-2}$) the gravitational acceleration and $A_{dg}$ ($m^2$) the degassing area (equal to the reactor horizontal cross section).

$$K_l.a = 2.39 \cdot 10^{-4} \left( \rho.g \frac{F_g}{A_{dg}} \right)^{0.86} \tag{3}$$

Upon increasing the dissolved oxygen concentration we found a decrease in required $F_g$ from 2.52 x $10^{-4}$ $m^3$ $s^{-1}$ to 0.70 x $10^{-4}$ $m^3$ $s^{-1}$ and this means a decrease by a factor of 3.6. Such a decrease could greatly reduce energy requirement for

large-scale cultivation of microalgae; however, this strategy would only be possible though in case $CO_2$-rich (flue) gas can be used in combination with a reactor design aiming at reduced light levels on the light-exposed surface (spatial dilution of light).

## 2.4. Conclusions

*Neochloris oleoabundans* appears to tolerate high $P_{O_2}$ levels at sub-saturating light since no decrease was observed upon changing $P_{O_2}$ from 0.21 to 0.63 bar. At $P_{O_2}$ =0.84 bar a decrease of growth was found under the conditions studied. At sub-saturating light intensities, photorespiration appeared to be the main cause of growth inhibition because restoring the $O_2/CO_2$ ratio through increasing $P_{CO_2}$, could offset the negative impact of elevated $P_{O_2}$. A similar strategy can reduce the energy input needed for oxygen removal. This approach could be used for production in a closed system (using diluted light) with lower energy requirements for degassing.

## 2.5. Acknowledgements

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## 2.6. References

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnology Advances, 25, 294-306.

Cuaresma, M., Janssen, M., Vilchez, C., Wijffels, R.H., 2011. Horizontal or vertical photobioreactors? How to improve microalgae photosynthetic efficiency. Bioresource Technology., 102, 5129-5137.

Dismukes, G.C., Carrieri, D., Bennette, N., Ananyev, G.M., Posewitz, M.C., 2008. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Current Opinion in Biotechnology, 19, 235-240.

Dubinsky, Z., Falkowski, P.G., Wyman, K., 1986. Light harvesting and utilization by phytoplankton. Plant and Cell Physiology, 27, 1335-1349.

Evers, E.G., 1991. A model for light-limited continuous cultures: growth, shading, and maintenance. Biotechnology and Bioengineering, 38, 254-259.

Giordano, M., Beardall, J., Raven, J.A., 2005. $CO_2$ Concentrating Mechanisms in Algae: Mechanisms, Environmental Modulation, and Evolution. Annual Review Plant Biology, 56, 99-131.

Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana hustedt*, and *Detonula confervagea (Cleve) gran*. Canadian Journal of Microbiology, 8, 229-239.

Guiry, W., 2011. In Guiry, M.D. and Guiry, G.M. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org; searched on 10 October 2011.

Kliphuis, A.M.J., de Winter, L., Vejrazka, C., Martens, D.E., Janssen, M., Wijffels, R.H., 2010. Photosynthetic efficiency of *Chlorella sorokiniana* in a turbulently mixed short light-path photobioreactor. Biotechnology Progress, 26, 687-696.

Kliphuis, A.M.J., Martens, D.E., Janssen, M., Wijffels, R.H., 2011. Effect of $O_2$:$CO_2$ ratio on the primary metabolism of *Chlamydomonas reinhardtii*. Biotechnology and Bioengineering, 108 (10), 2390-2402.

Molina, E., Fernández, F.G.A., Chisti, M.Y., 2001. Tubular photobioreactor design for algal cultures. Journal of Biotechnology, 92, 113-131.

Murata, N., Takahashi, S., Nishiyama, Y., Allakhverdiev, S.I., 2007. Photoinhibition of photosystem II under environmental stress. Biochimica et Biophysica Acta, 1767, 414-421.

Norsker, N.H., Barbosa, M.J., Vermuë, M.H., Wijffels, R.H., 2010. Microalgal production - A close look at the economics. Biotechnology Advances, 29, 24-27

Ogren, W.L., 1984. Photorespiration: Pathways, regulation, and modification. Annual Review Plant Physiology, 35, 415-442.

Osmond, C.B., 1981. Photorespiration and photoinhibition. Some implications for the energetics of photosynthesis. Biochimica Biophysica Acta, 639, 77-98.

Pospíšil, P., 2011. Molecular mechanisms of production and scavenging or reactive oxygen species by photosystem II. Biochimica Biophysica Acta, doi:10.1016/j.bbabio.2011.05.017.

Pruvost, J., Van Vooren, G., Cogne, G., Legrand, J., 2009. Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. Bioresource Technology, 100, 5988-5995.

Raso, S., Vermuë, M.H., Wijffels, R.H.,. Effect of oxygen concentration on the growth of *Nannochloropsis np.* at low light intensity. Journal of Applied Phycology, 1-9.

Raven, J.A., Giordano, M., Beardall, J., 2008. Insights into the evolution of CCMs from comparisons with other resource acquisition and assimilation processes. Physiology Plantarum, 133, 4-14.

Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R., 2009. Microalgae for Oil: Strain Selection, Induction of Lipid Synthesis and Outdoor Mass Cultivation in a Low-Cost Photobioreactor. Biotechnology and Bioengineering, 102, 100-112.

Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U., Mussgnug, J.H., Posten, C., Kruse, O., Hankamer, B., 2008. Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. Bioenergy Research, 1, 20-43.

Sierra, E., Acién, F.G., Fernández, J.M., García, J.L., González, C., Molina, E., 2008. Characterization of a flat plate photobioreactor for the production of microalgae. Chemical Engineering Journal, 138, 136-147.

Torzillo, G., Bernardini, P., Masojidek, J., 1998. On-line monitoring of chlorophyll fluorescence to assess the extent of photoinhibition of photosynthesis induced by high oxygen concentration and low temperature and its effect of the productivity

of outdoor cultures of *Spirulina platensis* (Cyanobacteria). Journal of Phycology, 34, 504-510.

Triantaphylides, C., Krischke, M., Hoeberichts, F.A., Ksas, B., Gresser, G., Havaux, M., Van Breusegem, F., Mueller, M.J., 2008. Singlet oxygen is the major reactive oxygen species involved in photooxidative damage to plants. Plant Physiology, 148, 960-968.

Ugwu, C.U., Aoyagi, H., Uchiyama, H., 2007. Influence of irradiance, dissolved oxygen concentration, and temperature on the growth of *Chlorella sorokiniana*. Photosynthetica, 45, 309-311.

Vance, P., Spalding, M.H., 2005. Growth, photosynthesis, and gene expression in *Chlamydomonas* over a range of $CO_2$ concentrations and $CO_2/O_2$ ratios: $CO_2$ regulates multiple acclimation states. Canadian Journal of Botany, 83, 796-809.

Wijffels, R.H., Barbosa, M.J., 2010. An outlook on microalgal biofuels. Science, 330, 913-913.

Wijffels, R.H., Barbosa, M.J., Eppink, M.H.M., 2010. Microalgae for the production of bulk chemicals and biofuels. Biofuels Bioproducts and Biorefining, 4, 287-295.

## Appendix 2A. Distribution of incident photon flux density over photobioreactor surface

In the experimental set-up used performed the incident light intensity varied over the illuminated surface. The photon flux density peaked at 350 µmol $m^{-2}$ $s^{-1}$ when facing the light source; was as low as 100 µmol $m^{-2}$ $s^{-1}$ when facing the reflective screens. The average value for the different experiments was always between 187 and 210 µmol $m^{-2}$ $s^{-1}$.

***Table A1*** *- Incident light measured at different radial positions and different heights of the photobioreactor prior to one of the experiments. All numbers represent photon flux densities in µmol PAR photons $m^{-2}$ $s^{-1}$.*

| | Radial Axis | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Height | a | b | c | d | e | f | g | h |
| 1 (0 cm) | 262 | 160 | 105 | 173 | 252 | 154 | 90 | 150 |
| 2 (4 cm) | 334 | 167 | 112 | 177 | 288 | 161 | 92 | 186 |
| 3 (8 cm) | 353 | 175 | 114 | 187 | 195 | 171 | 93 | 173 |
| 4(12cm) | 360 | 174 | 107 | 174 | 295 | 169 | 87 | 190 |
| Average | 187 | | | | | | | |

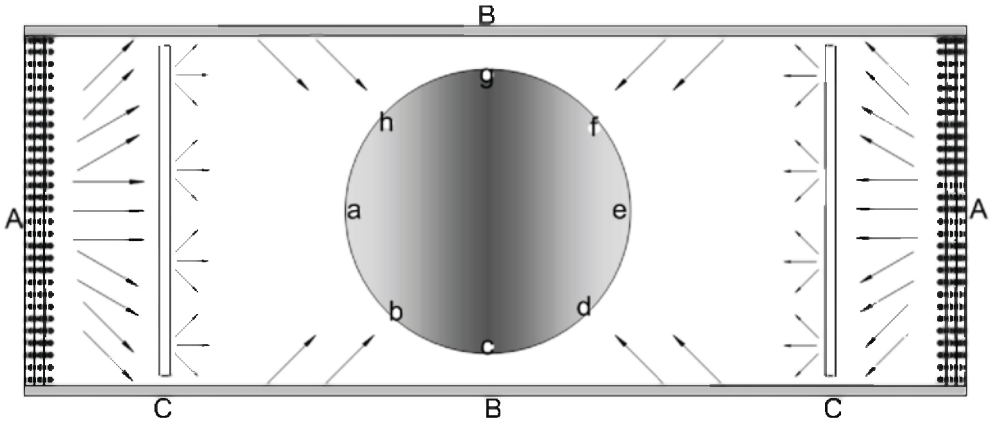


***Figure A1*** *- Top view illustrating the illumination of the bioreactor*
*A = LED panels, B = screens with reflective material, C = opal glass, a,b,c,d,e,f,g,h = radial positions for incident light measurement.*

***Table A2** - Photon flux density for each experimental run.*

| $P_{O_2}$ (bar) | Light intensity (µmol $m^{-2}$ $s^{-1}$) ± Stdev |
|---|---|
| 0.21 | 189 ± 83 |
| 0.63 | 187 ± 78 |
| 0.84 | 210 ± 71 |

## Appendix 2B. Calculation of light gradient in photobioreactor

The biomass specific absorption coefficient at a wavelength of 635 nm was used in the calculation, since this was the dominant wavelength of the LED light source used. The light gradient inside the bioreactor was estimated using Beers' law and the geometrical relationship derived for cylindrical vessels (Evers, 1991) with a small modification to account for the use of a flat cosine receiver as light sensor. This relation was modified to obtain the photon flux density on a flat ($2\pi$) cosine receiver facing the reactor wall ($PFD(z)_{wall}$) and facing the reactor centre ($PFD(z)_{centre}$) equations 1, 2 and 3. Where X (g $m^{-3}$) stands for biomass concentration; Θ is the angle of light path with line trough vessel center; α ($m^2$ g) the absorption coefficient; r (m) the vessel radius and s (m) the distance from vessel surface.

1

$$PFD(z)wall = \frac{PFDin}{\int_{0.5\pi}^{1.5\pi} \cos(\Theta+\pi)d\Theta} \cdot \left[ \int_{0.5\pi}^{1.5\pi} \cos(\Theta+\pi).\exp\left[ -\alpha.X.\left[ (r-s).\cos(\Theta) + \left[ r^2 - (r-s)^2.\sin(\Theta)^2 \right]^{0.5} \right] \right] d\Theta \right]$$

2

$$PFD(z)center = \frac{PFDin}{\int_{-0.5\pi}^{0.5\pi} \cos(\Theta+\pi)d\Theta} \cdot \left[ \int_{-0.5\pi}^{0.5\pi} \cos(\Theta+\pi).\exp\left[ -\alpha.X.\left[ (r-s).\cos(\Theta) + \left[ r^2 - (r-s)^2.\sin(\Theta)^2 \right]^{0.5} \right] \right] d\Theta \right]$$

3

$$PFD(z) = PFD(z)wall + PFD(z)center$$

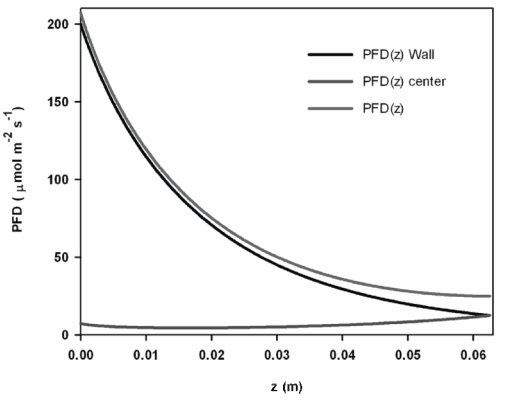


***Figure B1*** *- The PAR photon flux density on a flat (2π) cosine receiver facing the reactor wall ($PFD(z)_{wall}$) and facing the reactor centre ($PFD(z)_{centre}$) as a function of the depth z inside the cylindrical photobioreactor used. PFD(z) is the sum of both.*

## Appendix 2C. Specific absorption spectrum *N. oleoabundans*

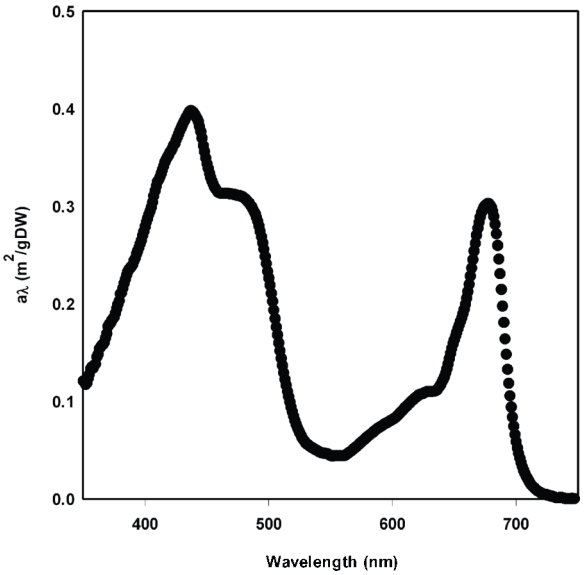


***Figure C1*** *- The wavelength dependent specific absorption coefficient of N. oleoabundans culture grown at 200 µmol $m^{-2}$ $s^{-1}$.*

# Chapter 3. EFFECT OF OXYGEN AT LOW AND HIGH LIGHT INTENSITY ON THE GROWTH OF *NEOCHOLORIS OLEOABUNDANS*

Claudia Sousa[1, 2], Ana Compadre[1], Marian H. Vermuë[2] and Rene H. Wijffels[2]

[1] Wetsus, P.O. Box 1113, 8900 CC Leeuwarden, the Netherlands

[2] Bioprocess Engineering, Wageningen University, P.O. Box 8129, 6700 EV, Wageningen, the Netherlands

**Abstract -** The effect of partial oxygen pressure on growth of *Neochloris oleoabundans* was studied at near-saturating light intensity in a fully-controlled photobioreactor. At the partial oxygen pressures tested ($P_{O_2}$= 0.24; 0.42; 0.63; 0.84 bar), the specific growth rate was 1.36; 1.16; 0.93 and 0.68 $day^{-1}$, respectively. An increase of the $P_{CO_2}$ from 0.007 to 0.02 bar at $P_{O_2}$ of 0.84 bar did not show any positive effect on the overall growth of the algae, contrary to what happens at sub-saturating light intensities. These results indicate that at near-saturating light intensity the inhibitory effect of oxygen by photorespiration cannot be overcome. The chlorophyll content of *Neochloris oleoabundans* grown at 200 µmol $m^{-2}$ $s^{-1}$ is about 1.9 times higher than when cultivated at 500 µmol $m^{-2}$ $s^{-1}$, whereas the carotenoid content was about 1.5 lower, both demonstrating photoacclimation effects. The elevated oxygen concentration in the growth medium does not affect the pigment content both at sub- and near-saturating light conditions. This indicates that elevated oxygen concentrations in the medium does not contribute to photo oxidative damage at the light conditions that are predominantly experienced by algae in closed photobioreactors, but only inhibit the growth via photo respiration effects.

**Key words:** *Neochloris oleoabundans*, oxygen inhibition, photooxidative damage, photoacclimation, photorespiration, photobioreactor

*Sousa, C., Compadre, A., Vermuë, M.H., Wijffels, R.H. 2013. Effect of oxygen at low and high light intensities on the growth of Neochloris oleabundans. Algal Research, 2 (2), 122-126.*

## 3.1. Introduction

*Neochloris oleoabundans* is one of the algae which combines high specific growth rate at optimal growth conditions (Gouveia et al., 2009; Li et al., 2008; Pruvost et al., 2009) with accumulation of lipids with large content of saturated fatty acids during nitrogen starvation conditions (Pruvost et al., 2009; Santos et al., 2012; Tornabene et al., 1983). These characteristics make this alga species a promising feed stock for biofuel production (Chisti, 2007; Schenk et al., 2008; Wijffels et al., 2010). For large-scale outdoor production of algae, closed photo-bioreactor systems (PBR) have been proposed. To make the production economically feasible, however, bottlenecks still need to be overcome. One of these bottlenecks is the high energy input that is required for mixing to provide the algae with light, carbon dioxide and to remove the photosynthetically produced oxygen (Dismukes et al., 2008; Norsker et al., 2011; Wijffels et al., 2010).

In photo-bioreactors oxygen that is produced during photosynthesis, accumulates and induces processes like photorespiration and photoinhibition, both leading to a decrease in biomass yield on light energy of the microalgae (Torzillo et al., 1998). In photorespiration, oxygen binds with the enzyme Rubisco and competes with carbon dioxide needed for photosynthesis. Hence, high oxygen levels lead to lower $CO_2$ uptake and reduced fixation of light energy into carbohydrates (Bader et al., 2000).

Photoinhibition occurs mainly at high and over-saturating light intensities. At those conditions an excess of electrons is generated in Photosystem II and these electrons will react with the photosynthetically produced oxygen, leading to the formation of oxygen radicals and other reactive oxygen species (ROS) such as $H_2O_2$ (Murata et al., 2007). In addition, light stimulates the formation of the highly reactive singlet oxygen via photo-activation (Triantaphylides et al., 2008). The singlet oxygen causes damage of the water-oxidizing center and deactivates the electron transport chain (Krieger-Liszkay et al., 2008; Hakala et al., 2005) and this results in loss of photosynthetic activity and death of cells.

To overcome the photo-oxidative damage caused by photoinhibition at high light, the microalgae have developed several protection mechanisms, generally referred to as photo-acclimation. Photo-acclimation can easily be recognized by changes in

the pigmentation of the algae, resulting in lower chlorophyll content and higher carotenoid content of the algae when exposed to higher irradiance. The carotenoids content normally increases to enable the algae to dissipate energy of excited chlorophyll and eliminate ROS and to maintain the photosystem structure (Demmig-Adams & Adams, 2002). In addition, carotenoids scavenge triplet chlorophyll and quench singlet oxygen (Falkowski & Raven, 2007). At very high light irradiation, however, the protective mechanisms cannot sufficiently deal with the surplus of electrons and formation of singlet oxygen and the accumulation of ROS occurs, leading to cell damage (Triantaphylides et al., 2008).

Although the combined effect of oxygen and light have been described in detail, the effect of accumulating oxygen on algal growth is only studied independently at controlled low light conditions (Kliphuis et al., 2011; Raso et al., 2011; Sousa et al., 2012). Upon an increase in oxygen concentrations in the algal cultures a general decrease in specific growth rates has been observed. This inhibition at low light conditions is related to the carboxylation/oxygenation ratio of the enzyme Rubisco and its affinity for oxygen and the oxygen inhibition effects at low light conditions can be compensated by an increase of the carbon dioxide concentration (Sousa et al., 2012). In outdoor cultivation; however, algae will experience different light conditions. It is thus important to know how the algae respond on accumulated oxygen at controlled culture conditions at higher light intensities and investigate if addition of carbon dioxide could be used to overcome the inhibiting effects of oxygen at higher light conditions as well. In this paper, the effect of oxygen partial pressure on the growth of *Neochloris oleoabundans* exposed to near-saturating conditions was determined in a fully-controlled photo-bioreactor operated in turbidostat mode and compared with the inhibiting effects of oxygen on growth at low light conditions. The magnitude of this effect on the specific growth rate as well as on the biomass yield on light energy and pigment content was determined. Finally, the $CO_2/O_2$ ratio was increased to see if the inhibiting effect of $O_2$ in microalgae could be overcome.

## 3.2. Material and methods

### 3.2.1. Cultures and medium

Adapted f/2 medium (Guillard & Ryther, 1962) was used to grow and maintain *Neochloris oleoabundans* (UTEX 1185) cultures. The medium was composed of artificial sea water (in mM): NaCl, 419; $MgCl_2.6H_2O$, 48.2; $CaCl_2.2H_2O$, 3.6; $Na_2SO_4$, 22.5; $K_2SO_4$, 4.9. The artificial sea water was enriched with the following nutrients (in mM): $NaH_2PO_4.2H_2O$, 2.50; $NaNO_3$, 32; trace elements (in µM): EDTA-FeNa, 29.3 $CuSO_4.5H_2O$, 0.10; $Na_2MoO_4.2H_2O$, 0.07; $ZnSO_4.7H_2O$, 0.19; $CoCl_2.6H_2O$, 0.19; $MnCl_2.4H_2O$, 2.27; vitamins (µg $L^{-1}$): thiamine, 200; biotine, 1.00; cyanocobalamine, 1.00.The pH was adjusted to 7.8 with 0.5 M NaOH. *Neochloris oleoabundans* was pre-cultured in an incubator with orbital shaker (Innova 44R, New Brunswick Scientific, USA) under fluorescent light (40 µmol $m^{-2}$ $s^{-1}$) at 25 °C and 120 rpm. The air inside the incubator was enriched with 2% carbon dioxide. In the reactor experiments the culture media was enriched with 10 mM $NaHCO_3$.

### 3.2.2. Photobioreactor

A 3 L jacketed bioreactor (Applikon Biotechnology, The Netherlands) was used to perform continuous turbidostat experiments. All sensors and regulators of the experimental set-up were connected to an Ez-controller equipped with Bioexpert© software (Applikon Biotechnology, The Netherlands). The culture was illuminated with two light panels (20×20 cm) with red (627 nm) LED lights (SL3500, Photon Systems Instruments, Czech Republic). The incident light intensity was measured with a PAR quantum sensor (model SA-190, LiCor Biosciences, USA) before the start of each experimental run. The measurements were done at different heights and radial positions to determine the average incident photon flux density ($PFD_{avg}$). The average value for the different experiments at high-light intensity was always ~ 500 µmol $m^{-2}$ $s^{-1}$, while at low light intensity the average incident photon flux density was ~200 µmol $m^{-2}$ $s^{-1}$ The measured and controlled process parameters were: pH, temperature, oxygen and carbon dioxide partial pressure in the liquid phase ($P_{O_2}$ and $P_{CO_2}$), liquid level, stirrer speed and optical density (OD) (Sousa et al., 2012).

The cells were adapted to the turbidostat conditions for at least 3 days, before the specific growth rate (μ) was determined from the dilution rate. The optical density at 750 ($OD_{750}$) and 680 nm ($OD_{680}$) was measured in a UV-visible spectrophotometer (UV-1650 PC, Schimadzu). The cell dry weight concentration and pigments content were determined off line as well.

### 3.2.3. Dry weight concentration

To determine the dry weight concentration, 5 mL samples in triplo were washed with 10 mL of ammonium formate 0.5 M, filtered through a pre-weighed glass fiber filter (Whatman GF/F), and washed again with 40 mL of ammonium formate 0.5 M. The filters were dried in an oven at 95 °C, for 24 h, in aluminium trays, cooled in a desiccator for at least 2 hours, and then weighed on a 5 digit analytical balance (ME235P-SD, Sartorius, Germany).

### 3.2.4. Chlorophyll and carotenoids

Chlorophyll and carotenoid content of the algae was determined in triplicate at the end of each experiment. A 2 mL algal aliquot collected from the reactor was centrifuged at 3760 rpm and 4 °C during 10 min (Allegra™ X-12 R Centrifuge). The pellets were frozen at - 80 °C, prior to further analysis. Chlorophyll was extracted by the adding 5 ml of methanol (100 %) to the biomass pellet. The cells were disrupted by ultrasound (Sonorex Digitec, Bandelin) combined with temperature shock (incubation at 60 °C and 0 °C). The suspension was centrifuged at 3760 rpm and 4 °C during 10 min. The supernatant was collected and chlorophyll and carotenoid content were determined at 470, 652 and 665 nm in a UV-visible spectrophotometer (UV-1650 PC, Schimadzu). Modified Arnon's equations (Lichtenthaler, 1987) were used to calculate chlorophyll and carotenoids concentrations in the extracts (Cuaresma et al., 2011). Chlorophyll and carotenoid content were presented per gram of biomass which was calculated based on the dry weight concentrations in the samples used.

## 3.3. Results and discussion

### 3.3.1. Controlled cultivation of algae at high and low light intensity

Figure 1 shows a typical run at 0.21 bar of oxygen partial pressure of *Neochloris oleobundans* cultivated at high light conditions. In this experiment the algae were cultivated using an average incident light irradiance of 500 µmol $m^{-2}$ $s^{-1}$. The accompanying average photon flux density experienced by the algae inside the photobioreactor was 230 µmol $m^{-2}$ $s^{-1}$, using an estimated light gradient inside the photobioreactor, as was described by Sousa et al. (2012). The PI (photosynthesis-irradiance) curve for this alga (Sousa et al., 2012) shows that *Neochloris oleoabundans* experiences near-saturation conditions at 230 µmol $m^{-2}$ $s^{-1}$. When growing the algae at an average low incident light intensity of 200 µmol $m^{-2}$ $s^{-1}$, they experience 92 µmol $m^{-2}$ $s^{-1}$ inside the photobioreactor which corresponds with sub-saturating light conditions according to the PI curve.

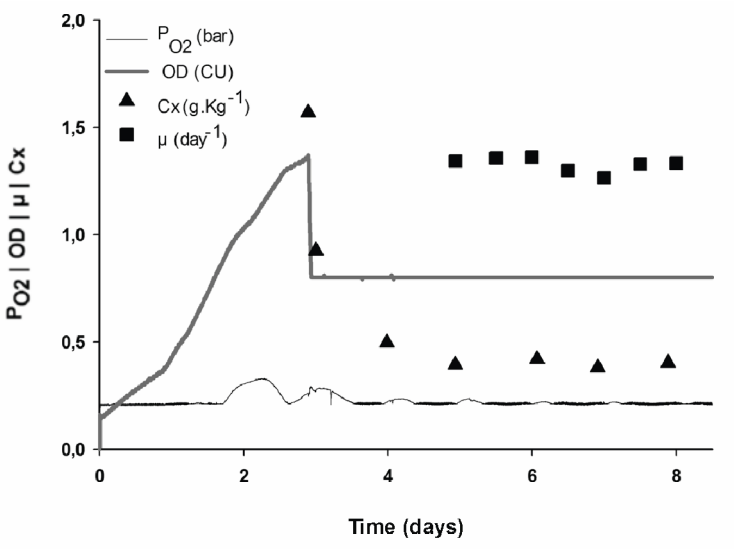


***Figure 1*** *- Graphical representation of the partial oxygen pressure ($P_{O_2}$), optical density (OD), dry weight concentration ($C_x$), pH and specific growth rate (µ) of Neochloris oleoabundans in time at incident light intensity of 500 µmol $m^{-2}$ $s^{-1}$.*

After a batch period of 3 days the algae culture reached an OD of 1.4 CU and the turbidostat operation was started. During the first day of turbidostat cultivation

the optical density decreased to a constant set level of 0.8 CU that corresponds to a biomass concentration of 0.42 ± 0.05 gDW $L^{-1}$ (Fig. 1, Table 1) while the specific growth rate (μ) remained constant at 1.36 $day^{-1}$.

### 3.3.2. Oxygen effects of microalgal growth at high and low light intensity

The cultivation of *Neochloris oleoabundans* was conducted at 4 different oxygen concentrations at high light conditions. Table 1 shows the results of the experiments performed. The results obtained at low incident light intensity (200 μmol $m^{-2}$ $s^{-1}$) were obtained from Sousa et al. (2012) and added for comparison.

***Table 1*** *- Specific growth rate (μ), and biomass concentration ($C_x$) of the microalgae Neochloris oleoabundans at different partial oxygen and carbon dioxide pressures at high (500 μmol $m^{-2}$ $s^{-1}$) incident light intensity. The results obtained at low incident light intensity (200 μmol $m^{-2}$ $s^{-1}$) were obtained from Sousa et al. (2012)*

| $NaHCO_3$ (mM) | $P_{O_2}$ (bar) | $P_{CO_2}$ (bar) | μ ($day^{-1}$) ± Stdev | $C_x$ (g $kg^{-1}$) ± Stdev | μ ($day^{-1}$) ± Stdev | $C_x$ (g $kg^{-1}$) ± Stdev |
|---|---|---|---|---|---|---|
| | | | 500 μmol $m^{-2}$ $s^{-1}$ | | 200 μmol $m^{-2}$ $s^{-1}$ | |
| 10 | 0.21 | 0.007 | 1.36 ± 0.20 | 0.42 ± 0.05 | 1.38 ± 0.17 | 0.40 ± 0.002 |
| 10 | 0.42 | 0.007 | 1.16 ± 0.26 | 0.42 ± 0.05 | - | - |
| 10 | 0.63 | 0.007 | 0.93 ± 0.14 | 0.42 ± 0.02 | 1.36 ± 0.18 | 0.41 ±0.04 |
| 10 | 0.84 | 0.007 | 0.68 ± 0.28 | 0.43 ± 0.03 | 1.06 ± 0.02 | 0.39 ± 0.004 |
| 30 | 0.84 | 0.020 | 0.68 ± 0.15 | 0.39 ± 0.05 | 1.36 ± 0.002 | 0.39 ± 0.004 |

This table shows that the specific growth rates decrease with an increase of the partial oxygen pressure. The highest growth rate value at 500 μmol $m^{-2}$ $s^{-1}$ (1.36 $day^{-1}$) was obtained for *Neochloris oleoabundans* cultivated at 0.21 bar partial oxygen pressure, while at 0.84 bar partial oxygen pressure the growth rate was reduced by almost 50%. Previous work at low incident light conditions of 200 μmol $m^{-2}$ $s^{-1}$ (Sousa et al., 2012) had shown that the inhibiting effect of oxygen could be overcome by addition of extra $CO_2$ indicating that oxygen mainly inhibited the algae via respiration. In this experiment at near-saturating light conditions, the bicarbonate concentration ($NaHCO_3$) was increased from 10 to 30 mM as well. The higher bicarbonate concentration, however, did not result in an increase of the specific growth rate. While an increase of the bicarbonate

concentration proved to be an efficient method to diminish inhibition of oxygen via photorespiration at low light conditions, it did not have any effect on growth at the high light conditions used in this experiment.

The increase of bicarbonate and consequently the increase of $CO_2$ to the culture medium did not help to reduce the effects of oxygen on the specific growth rate at high light conditions. It is possible that the effect of additional $CO_2$ in the medium culture did not contribute enough to the overcome the inhibitory effect by photorespiration. At high light intensities, the local concentration of oxygen at the Rubisco site is higher and the $CO_2$ is lower than in the medium culture. The addition of $CO_2$ in the medium at these conditions might not be effective enough to change the local ratio $CO_2/O_2$ in the vicinity of the Rubisco and there is hardly any effect by this addition. On top of the inhibitory effects of photorespiration also photoinhibition is expected to occur at high light conditions.

When cultivating other microalgal species like *Phaeodactylum tricornutum* (Molina et al., 2001); *Chlorella sorokiniana* (Ugwu et al., 2007), *Spirulina platensis* at high light intensity, a similar decrease in specific growth rate was observed at elevated oxygen concentrations. The described experiments, however, were not performed at controlled conditions of oxygen. It is therefore not possible to distinguish between the direct effects of light and of oxygen on the growth of the algae. In the present study, the experiment was performed at controlled partial oxygen pressures and irradiance. These studies claim that the cells phase photoinhibition effects, but it is not clear, if elevated oxygen levels in the medium stimulate photo-oxidation and thereby contribute to additional photo-inhibitory effects on growth.

### 3.3.3. Oxygen effects of pigment content at high and low light intensity

Figure 2 presents the chlorophyll content of *Neochloris oleoabundans* measured at different partial oxygen pressures at two different light conditions. The cells show photo-acclimation; since the average chlorophyll content of *N. oleoabundans* grown at low light intensity (200 µmol $m^{-2}$ $s^{-1}$) is about 1.9 times higher than when cultivated at high light intensity (500 µmol $m^{-2}$ $s^{-1}$). Photo-acclimation is a process in which the photosynthetic pigment content is reduced

as a protection mechanism of the photosynthetic apparatus against increased irradiance (Anemaet et al., 2010). Such a clear dependency of chlorophyll content per cell in response to different light intensities is a common mechanism among microorganisms that perform photosynthesis. Chlorophyll is a light-harvesting pigment that, under low light, increases until the cells become optically dark; and under high light, it decreases, resulting in cells rather transparent.

At both light intensities no effect of partial oxygen pressures on chlorophyll content was found. At 200 µmol $m^{-2}$ $s^{-1}$ the amount of chlorophyll ranged from 23.8 at $P_{O_2}$ = 0.63 bar to 28.2 mgChl $gDW^{-1}$ at $P_{O_2}$ = 0.84 bar, while at an incident light intensity of 500 µmol $m^{-2}$ $s^{-1}$, chlorophyll content ranged from 11.3 measured at $P_{O_2}$ = 0.42 bar till 16.2 mgChl $gDW^{-1}$ at $P_{O_2}$ = 0.63 bar.

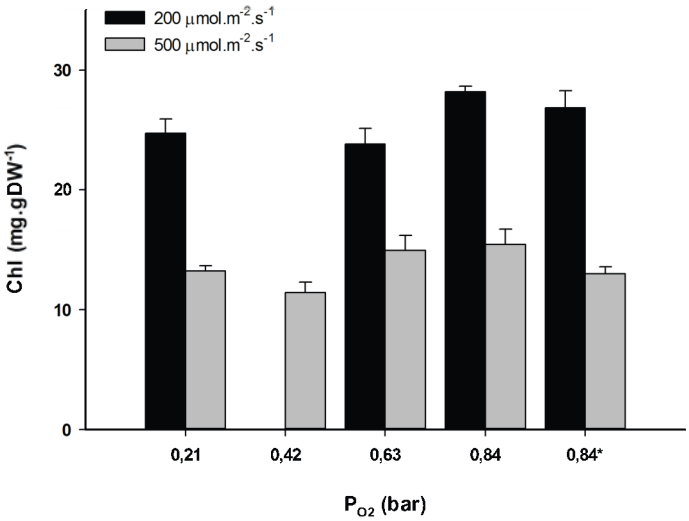


***Figure 2*** *- Chlorophyll content in Neochloris oleoabundans cultivated at sub-saturating light intensity (200 µmol $m^{-2}$ $s^{-1)}$ and near saturating light intensity (500 µmol $m^{-2}$ $s^{-1}$). At $P_{O_2}$=0.84 the bicarbonate concentration in the culture media was 10 mM and at $P_{O_2}$=0.84* the bicarbonate concentration was increased to 30 mM.*

Figure 2 shows that no significant effects of partial oxygen pressure and partial carbon dioxide pressure on the chlorophyll content was found at both light intensities; no loss or damage of chlorophyll seem to occur due to oxygen accumulation or increase of carbon dioxide. In general, the same increasing trend

of chlorophyll content upon a decrease of irradiance is reported for *Thallasiosira pseudonana* (Valenzuela-Espinoza et al., 2007) and *Spirulina platensis* (Anemaet et al., 2010). Chlorophyll concentrations in *Thallasiosira pseudonana* were 1.74 mg/L at low light intensity (50 µmol $m^{-2}$ $s^{-1}$) and 0.47 mg/L at high light irradiance (750 µmol $m^{-2}$ $s^{-1}$) (Valenzuela-Espinoza et al., 2007). During the growth of *Spirulina platensis*, the highest chlorophyll content (14.6 mg $g^{-1}$) was detected at incident light intensity of ~40 µmol $m^{-2}$ $s^{-1}$ and smallest chlorophyll contents (6.2 mg $g^{-1}$) were found when cultivating *Spirulina* under light intensity of 100 µmol $m^{-2}$ $s^{-1}$ in this study (Anemaet et al., 2010).

The accumulation of oxygen in the photobioreactor was expected to induce extra formation of oxygen radicals and singlet oxygen. Singlet oxygen damages protein in chloroplasts and inactivates electron transport mechanism (Ledford & Niyogi, 2005; Murata et al., 2007). The lifetime of singlet oxygen is rather short. Singlet oxygen thus reacts with the molecules in its vicinity such as pigments which are involved in the electron transport mechanism and proteins present in the chloroplast. Figure 2 shows, however that the chlorophyll was not damaged by the oxygen present in the medium, indicating that no additional singlet oxygen was formed at higher oxygen concentrations at the light conditions used.

Carotenoids are known to protect the cells against photo-inhibition at high light intensity and increased inhibition by photo-oxidation is accompanied by increased pigment content (Falkowski & Raven, 2007). Carotenoids play an important role in photosynthesis; located in the chloroplast they contribute to light harvesting, dissipation of light energy, scavenging of triplet chlorophyll and singlet oxygen, and maintaining the photosystem structure. They are claimed to be a major antioxidant defense (Demmig-Adams & Adams, 2002). Figure 3 shows that the carotenoid content of *Neochloris oleoabundans* grown at low light intensity (200 µmol $m^{-2}$ $s^{-1}$) is indeed lower than the carotenoid content of the cells cultivated at high light intensity (500 µmol $m^{-2}$ $s^{-1}$).

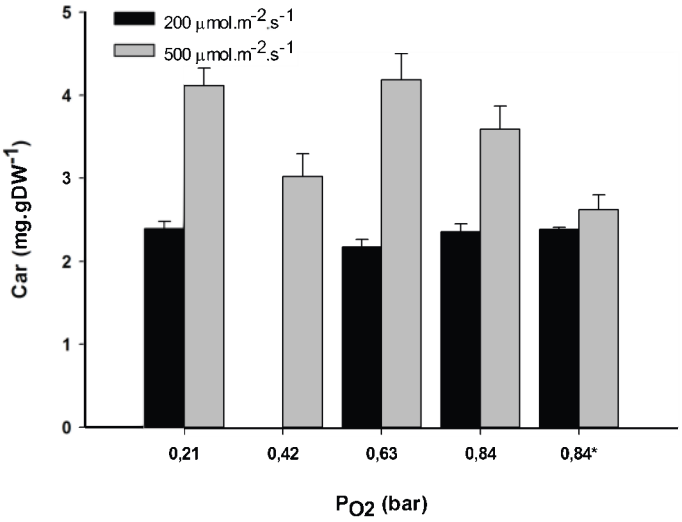


***Figure 3*** *- Carotenoids content in Neochloris oleoabundans cultivated at sub-saturating light intensity ( 200 µmol $m^{-2}$ $s^{-1}$) and near saturating light intensity ( 500 µmol $m^{-2}$ $s^{-1}$). At $P_{O_2}$=0.84 the bicarbonate concentration in the culture media was 10mM and at $P_{O_2}$=0.84* the bicarbonate concentration was increased to 30mM.*

At elevated oxygen concentrations in the photobioreactor, extra formation of oxygen radicals and singlet oxygen was expected and those would induce extra pigment production to protect the cells by quenching the electrons and act as anti-oxidant against the ROS formed. The carotenoid content in cells of *Neochloris oleoabundans* cultivated at elevated partial oxygen pressures, however, is hardly affected. At an incident light intensity of 200 µmol $m^{-2}$ $s^{-1}$ a maximum of 2.7 mgCar $gDW^{-1}$ was measured at a partial oxygen pressure of 0.84 bar and a minimum of 2.3 mgCar $gDW^{-1}$ at $P_{O_2}$ = 0.63 bar. At high light intensity (500 µmol $m^{-2}$ $s^{-1}$) the highest value is 4.9 mgCar $gDW^{-1}$ and the lowest 3.1 mgCar $gDW^{-1}$.

Vonshak et al. (1996) refers to photoinhibition as a time and light dependent decline in photosynthesis that happens in a first stage of the exposure of the algae to high light and oxygen and to photo-oxidation in a secondary stage leading to damage and/or death of the cells. Our results indicate that at this light intensity the cells were indeed exposed to photoinhibition but no relation between carotenoid content and oxygen concentration (figure 3) was found, indicating that

elevated oxygen concentrations in the medium do not cause additional photo-oxidative damage at sub-saturating and near-saturating light conditions.

## 3.4. Conclusions

High oxygen concentrations negatively affected the growth rate of *Neochloris oleoabundans* at high light conditions. At such conditions, photorespiration combined with photoinhibition of the culture is bound to occur, resulting in the deterioration of microalgae cell viability and the decrease in *Neochloris oleoabundans* growth rate. A 3 times increase in bicarbonate addition did not show any positive effect of the overall growth of the algae at near-saturating light contrary to what happens at sub-saturating light intensities. This indicates that addition of extra carbonate to the medium to overcome the photorespiration effects, is insufficient to compensate for the loss of biomass due to the combined photorespiration and photoinhibition at near-saturating light conditions. The elevated oxygen concentration in the growth medium did not affect chlorophyll and carotenoid content at sub- and near-saturating light conditions, indicating that the elevated oxygen concentration in the medium did not contribute to the photoinhibition effects experienced at the higher light intensities. These results indicate that the photoinhibition effects are only due to the increased irradiance used and not due to accumulation of the oxygen in the medium as such. The photoinhibition effects can thus only be prevented by working at sub-saturating light conditions rather than at near-saturating light conditions. For large-scale outdoor cultivation of micro-algae our results indicate that reactor configurations that allow spatial dilution of light should be used, in combination with addition of carbonate. In these types of photobioreactors the algae grow at sub-saturating light conditions and with the addition of carbon dioxide the photorespiration effects will be minimized. This reduces the need for degassing to remove the surplus of oxygen from the medium. In this way, the total energy and costs required for degassing can be decreased.

## 3.5. Acknowledgements

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## 3.6. References

Anemaet, I.D., Bekker, M., Hellingwerf, K.J. 2010. Algal photosynthesis as the primary driver for a sustainable development in energy, feed, and food production. Marine Biotechnology, DOI 10.1007/s10126-010-9311-1.

Bader, M.R., von Caemmerer, S., Ruuska, S., Nakano, H. 2000. Electron flow to oxygen in higher plants and algae: rates and control of direct photoreduction (Mehler reaction) and rubisco oxygenase. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences, 355(1402), 1433-1445.

Chisti, Y. 2007. Biodiesel from microalgae. Biotechnology Advances, 25, 294-306.

Cuaresma, M., Janssen, M., Vilchez, C., Wijffels, R.H. 2011. Horizontal or vertical photobioreactors? How to improve microalgae photosynthetic efficiency. Bioresource Technology, 102(8), 5129-5137.

Demmig-Adams, B., Adams, W.W. 2002. Antioxidants in Photosynthesis and Human Nutrition. Science, 298 (5601 ), 2149- 2153

Dismukes, G.C., Carrieri, D., Bennette, N., Ananyev, G.M., Posewitz, M.C. 2008. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Current Opinion in Biotechnology, 19(3), 235-240.

Falkowski, P.G., Raven, J.A. 2007. Aquatic photosynthesis. Princeton University Press, Princeton, NJ.

Gouveia, L., Marques, A.E., da Silva, T.L., Reis, A. 2009. *Neochloris oleabundans* UTEX #1185: a suitable renewable lipid source for biofuel production. Journal of Industrial Microbiology and Biotechnology, 1-6.

Guillard, R.R.L., Ryther, J.H. 1962. Studies of marine planktonic diatoms. *I. Cyclotella nana hustedt*, and *Detonula confervagea (Cleve) gran*. Canadian Journal for Microbiology, 8, 229-239.

Hakala, M., Tuominen, I., Keranen, M., Tyystjarvi, T., Tyystjarvi, E. 2005. Evidence for the role of the oxygen-evolving manganese complex in photoinhibition of Photosystem II. Biochimica Et Biophysica Acta-Bioenergetics, 1706(1-2), 68-80.

Kliphuis, A.M.J., Martens, D.E., Janssen, M., Wijffels, R.H. 2011. Effect of $O_2$:$CO_2$ ratio on the primary metabolism of *Chlamydomonas reinhardtii*. Biotechnology and Bioengineering, 108(10), 2390-2402.

Krieger-Liszkay, A., Fufezan, C., Trebst, A. 2008. Singlet oxygen production in photosystem II and related protection mechanism. Photosynthesis Research, 98(1-3), 551-564.

Ledford, H.K., Niyogi, K.K. 2005. Singlet oxygen and photo-oxidative stress management in plants and algae. Plant Cell and Environment, 28(8), 1037-1045.

Li, Y., Horsman, M., Wang, B., Wu, N., Lan, C.Q. 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Applied Microbiology and Biotechnology, 81(4), 629-636.

Lichtenthaler, H.K. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods in Enzymology, 148, 350-382.

Molina, E., Fernandez, F.G.A., Chisti, M.Y. 2001. Tubular photobioreactor design for algal cultures. Journal of Biotechnology, 92, 113-131.

Murata, N., Takahashi, S., Nishiyama, Y., Allakhverdiev, S.I. 2007. Photoinhibition of photosystem II under environmental stress. Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1767(6), 414-421.

Norsker, N.H., Barbosa, M.J., Vermuë, M.H., Wijffels, R.H. 2011. Microalgal production - A close look at the economics. Biotechnology Advances, 29(1), 24-27.

Pruvost, J., Van Vooren, G., Cogne, G., Legrand, J. 2009. Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. Bioresource Technology, 100(23), 5988-5995.

Raso, S., van Genugten, B., Vermuë, M., Wijffels, R.H. 2011. Effect of oxygen concentration on the growth of *Nannochloropsis sp.* at low light intensity. Journal of Applied Phycology, 1-9.

Santos, A.M., Janssen, M., Lamers, P.P., Evers, W.A.C., Wijffels, R.H. 2012. Growth of oil accumulating microalga *Neochloris oleoabundans* under alkaline-saline conditions. Bioresource Technology, 104, 593-599.

Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U., Mussgnug, J.H., Posten, C., Kruse, O., Hankamer, B. 2008. Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. Bioenergy Research, 1, 20-43

Sousa, C., de Winter, L., Janssen, M., Vermuë, M.H., Wijffels, R.H. 2012. Growth of the microalgae *Neochloris oleoabundans* at high partial oxygen pressures and sub-saturating light intensity. Bioresource Technology, 104, 565-570.

Tornabene, T.G., Holzer, G., Lien, S., Burris, N. 1983. Lipid composition of the nitrogen starved green alga *Neochloris oleoabundans*. Enzyme and Microbial Technology, 5, 435-440.

Torzillo, G., Bernardini, P., Masojidek, J. 1998. On-line monitoring of chlorophyll fluorescence to assess the extent of photoinhibition of photosynthesis induced by high oxygen concentration and low temperature and its effect on the productivity of outdoor cultures of *Spirulina platensis* (cyanobacteria). Journal of Phycology, 34 504 - 510

Triantaphylides, C., Krischke, M., Hoeberichts, F.A., Ksas, B., Gresser, G., Havaux, M., Van Breusegem, F., Mueller, M.J. 2008. Singlet oxygen is the major reactive oxygen species involved in photooxidative damage to plants. Plant Physiology, 148(2), 960-968.

Ugwu, C.U., Aoyagi, H., Uchiyama, H. 2007. Influence of irradiance, dissolved oxygen concentration, and temperature on the growth of *Chlorella sorokiniana*. Photosynthetica, 45(2), 309-311.

Valenzuela-Espinoza, E., Millan-Nunez, R., Trees, C.C., Santamaria-del-Angel, E., Nunez-Cebrero, F. 2007. Growth and accessory pigment to chlorophyll a ratios of *Thalassiosira pseudonana* (Bacillariophyceae) cultured under different irradiances. Hidrobiologica, 17(3), 249-255.

Vonshak, A., Torzillo, G., Accolla, P., Tomaselli, L. 1996. Light and oxygen stress in *Spirulina platensis* (cyanobacteria) grown outdoors in tubular reactors. Physiologia Plantarum, 97(1), 175-179.

Wijffels, R.H., Barbosa, M.J., Eppink, M.H.M. 2010. Microalgae for the production of bulk chemicals and biofuels. Biofuels, Bioproducts and Biorefining, 4(3), 287-295.

# Chapter 4. EFFECT OF DYNAMIC OXYGEN CONCENTRATIONS ON THE GROWTH OF *NEOCHOLORIS OLEOABUNDANS* AT SUB-SATURATING LIGHT CONDITIONS

Claudia Sousa[1, 2], Dimitar Valev[1,2], Marian H. Vermuë[2] and Rene H. Wijffels[2]

[1] Wetsus, P.O. Box 1113, 8900 CC Leeuwarden, the Netherlands

[2] Bioprocess Engineering, Wageningen University, P.O. Box 8129, 6700 EV, Wageningen, the Netherlands

**Abstract** - Tubular photobioreactors for micro-algae production are considered economically-feasible, but only if the energy needed to remove the photosynthetically produced oxygen can be reduced considerably. In this study, the effects of the increase of the oxygen concentration followed by a decrease of the oxygen in the degasser were simulated in a CSTR at fully controlled conditions at sub-saturating light intensity and the effect of a 10 times elongation of the residence time at in the solar receiver was investigated. Therefore 3 different light regimes were used: continuous light; 30 minutes light on followed by 6 minutes light off and 300 minutes light on followed by 6 minutes light off. The specific growth rate measured at constant low oxygen concentration $P_{O_2}$ = 0.21 bar during these three light regimes were 1.14 ± 0.06, 0.80 ± 0.16 and 1.09 ± 0.05 $day^{-1}$ respectively. The effect of dynamically changing oxygen concentrations from $P_{O_2}$ = 0.21 bar to $P_{O_2}$ = 0.63 bar followed by subsequent degassing to $P_{O_2}$ = 0.21 bar during the dark period resulted in similar specific growth rates. The decrease of the algae specific growth observed when applying different light regimes, shows that the exposure of the algae cells to dark periods in the degasser has bigger negative impact than the temporary exposure to accumulating oxygen concentrations in the solar receiver. Based on the observed algae physiology under dynamic oxygen concentration, reducing the number of degassing units and increasing their degassing capacity will result in substantial savings in capital and energy costs.

**Key words:** tubular photobioreactors, dynamic oxygen, light regime, *Neochloris oleoabundans*

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## 4.1. Introduction

Lipid-rich microalgae such as *Neochloris oleoabundans* are considered to be a renewable resource for biofuel production (Wijffels et al., 2010). Although these microalgae show high growth rates and high lipid content, large-scale outdoor production of microalgae is still not economically feasible. The feasibility study of Norsker et al. (2011) shows that a tubular photobioreactor (PBR) can be used as an economically-feasible system for production of algae, but only if the energy consumption is considerably reduced. The current main bottleneck in this production system is the energy needed for circulation of the liquid through the tubes of the photobioreactor and for exchange of gases in the degasser (Norsker et al., 2011). This circulation and gas exchange is needed to supply the algae with light, $CO_2$ and to remove the oxygen produced.

The oxygen needs to be removed as it causes photorespiration. In photorespiration $O_2$ competes with $CO_2$ for the key enzyme in the Calvin cycle, RuBiSco, which is responsible for incorporating inorganic carbon in organic molecules, resulting in less growth of the algae and lower biomass yield (Foyer et al., 2009; Miron et al., 1999). Another process which limits microalgae growth is photoinhibition. This process, however, mainly occurs at near- and over-saturating light intensities and is hardly relevant at sub-saturating light intensities (Kliphuis et al., 2011; Raso et al., 2011; Sousa et al., 2012).

Previous work on the effect of oxygen at sub-saturating light on microalgae growth showed the expected decrease in specific growth rate with increasing oxygen concentration (Kliphuis et al., 2011; Raso et al., 2011; Sousa et al., 2012). All studies mentioned were performed at constant oxygen concentrations. In practice, however, the algae cultured in closed tubular photobioreactor systems experience an increasing oxygen concentration gradient along the length of the tubes, and this may induce a decreasing growth rate along the tubes (Ugwu et al., 2008). In addition, a non-illuminated degassing unit is placed at the end of the tube to remove the accumulated oxygen. In this study the effect of dynamic changing oxygen concentrations on the algal growth in photobioreactors will be evaluated, also taking the effect of the residence time of the algae in the dark degasser into account. In addition, the effect of extension of the exposure time to high oxygen concentrations will be investigated. A possible extension of this

residence time would mean that less degassing units are needed and this would result in a lower overall power consumption for degassing combined with higher overall productivity in the system, as the algae spend relatively little time in the dark.

To mimic the dynamic changes in oxygen concentration occurring in tubular systems, a fully controlled Continuous Stirred Tank Reactor (CSTR) operated in turbidostat mode was used to culture *Neochloris oleoabundans* at sub-saturating light intensity. During the turbidostat run, oxygen was allowed to build up from $P_{O_2}$ = 0.21 to 0.63 bar and then was rapidly decreased during a short time of darkness to the starting level while the specific growth rate was monitored. The experiments were repeated while extending the time at which the algae were exposed to high partial oxygen pressures in order to determine the effect on the specific growth rate of the algae.

## 4.2. Materials and methods

### 4.2.1. Cultures and medium

*Neochloris oleoabundans* (UTEX 1185) was cultured and maintained in 250 ml Erlenmeyer flasks in 100ml of adapted f/2 medium (Guillard & Ryther, 1962). The flasks were closed with porous stoppers (Bio-silico, Hirschmann Laborgeräte GmbH & Co.KG, Germany) and placed in an incubator with an orbital shaker (Innova 44R, New Brunswick Scientific, USA) under fluorescent light (40 µmol $m^{-2}$ $s^{-1}$) at 25 °C and 120 rpm. The air inside the incubator was enriched with 2% carbon dioxide. The medium used for the reactor runs was enriched with 10 mM $NaHCO_3$. Both media were filter-sterilized using 0.22 µm filters and kept at pH of 7.8.

### 4.2.2. Photobioreactor

A 3L jacketed bioreactor (Applikon Biotechnology, The Netherlands), equipped with marine impeller was operated in turbidostat mode. The illuminated surface of the reactor was 0.061 $m^2$. An Ez-controller operating with Bioexpert© software (Applikon Biotechnology, The Netherlands) was used for monitoring and control. The on-line measured process parameters were pH, temperature, partial oxygen

pressure in the liquid phase ($P_{O_2}$), liquid level, stirrer speed and optical density (OD). During the run the pH was controlled at pH 7.8 by automatic addition of gaseous carbon dioxide ($CO_2$), the temperature was maintained at 25 °C and the optical density (OD) was controlled by a turbidity sensor (ASD19-N, Optek, Germany) connected to a peristaltic medium pump to keep a constant optical density. The liquid level in the reactor was controlled at ~ 2L by a level sensor connected to a peristaltic pump for removing the excess of culture, which was activated when necessary. During the experimental run, dynamic oxygen concentrations were applied: the oxygen was allowed to build up to $P_{O_2}$ = 0.63 bar and then degassed to $P_{O_2}$ = 0.21 bar. The partial oxygen pressure was decreased to the desired levels by the automatic addition of gaseous dinitrogen ($N_2$). The partial oxygen pressure was monitored by a Clark type $P_{O_2}$ sensor (LowDrift sensor, Applisens, The Netherlands). Calibration of the sensor was performed inside the reactor with filtered adapted f/2 medium, before inoculation, using pure $O_2$ giving a partial oxygen pressure ($P_{O_2}$) of 1 bar. The cells were allowed to adapt to the turbidostat conditions, before the specific growth rate (μ) was determined from the dilution rate.

### 4.2.3. Light regime

Light was provided by two LED light panels (20x20 cm) (SL3500, Photon Systems Instruments, Czech Republic). The light sources were positioned at both sides of the reactor and a plate of opal glass was placed in front of each of the light panels, to ensure homogeneous light distribution. In addition, reflective material was placed around the reactor. A PAR quantum sensor (model SA-190, LiCor Biosciences, USA) was used to measure the average incident photon flux density ($PFD_{avg}$) on the reactor surface. The average value incident photon flux density was 198 μmol $m^{-2}$ $s^{-1}$. The PI (Photosynthesis-irradiance) curve for this algae shows that *Neochloris oleoabudans* indeed experiences sub-saturating light conditions at the measured $PFD_{avg}$ (Sousa et al., 2012).

The light sources were connected to a Siemens PLC Relay (LOGO!) light controller to simulate the dark period in the degasser of a tubular PBR system. Two different time regimes for the light were used, related with the oxygen conditions applied during the experiments. The first one was 30 minutes light “On” and 6 minutes

light "Off" (30/6 regime). This regime was applied during dynamic oxygen conditions, when the algae were allowed to produce and accumulate oxygen from $P_{O_2}$ = 0.21 bar up to $P_{O_2}$ = 0.63 bar. After this phase a degassing phase was initiated. During the degassing phase the light controller switches to Lights "Off" and rapid degassing of the liquid volume is initiated to bring the oxygen concentrations back to starting levels of $P_{O_2}$ = 0.21 bar in 6 minutes. The second time regime was operated at 300 minutes light "On" and 6 minutes light "Off" (300/6 regime). This regime was applied during cultivation of the algae at high oxygen levels ($P_{O_2}$ = 0.63 bar) for 300 minutes. The degassing phase was performed at six minutes lights "Off".

### 4.2.4. Dry weight concentration

Triplicate samples of 5 ml were collected on a daily base for dry weight determination. The samples were diluted with 10 ml ammonium formate (0.5M) and filtered over pre-weighed glass fibre-filters (Whatman GF/F). An additional 40 ml of ammonium formate (0.5M) was used for washing. Filters with biomass were dried at 95 °C for 24 hours in aluminium trays, cooled in desiccator for 2 hours and weighted on a 5 digit analytical balance (ME235P-SD, Sartorius, Germany).

### 4.2.5. Chlorophyll and Carotenoid determination

The dynamically changing oxygen concentrations but especially the different light-darkness regimes applied could cause additional photo-acclimation and photo-inhibition effects. Chlorophyll and carotenoids are both pigments for light harvesting and the latter serve as photo protective pigments to protect the photosynthetic machinery from excess of light by scavenging reactive oxygen species and singlet oxygen (Demmig-Adams & Adams, 2002; Falkowski & Raven, 2007; Triantaphylidès & Havaux, 2009; Vilchez et al., 2011). Although the effect of light on the carotenoid content is described in detail (Britton et al., 1999; Lamers et al., 2008) it is not known if higher oxygen concentrations in the medium affect the chlorophyll and carotenoid content as well. To verify if these photo-protective mechanisms were activated when the cells are subjected to dynamic oxygen concentrations, the chlorophyll and total carotenoids were determined. Triplicate samples were collected on a daily base and centrifuged at 3750 rpm for 10 min. at

4 °C and subsequently the pellets frozen at -80 °C. The extraction was done by addition of 5 mL of 100% methanol to each tube. The tubes were then placed for 5 min. in ultrasound bath (Sonorex Digitec, Bandelin). Afterwards the samples were incubated for 40 min at 60 °C and then for 15 min at 0 °C. The suspension was centrifuged once more at the same conditions (3750 rpm, 10 min. and 4 °C). The supernatant collected and chlorophyll and carotenoid determined at 470 nm, 652 nm and 665 nm in a UV-visible spectrophotometer (UV_1650 PC, Schimadzu). The equations used to determine the chlorophyll and carotenoid content were modified Arnon's equations (Lichtenthaler, 1987). Chlorophyll and carotenoid content were expressed per gram of biomass, calculated based on the dry weight concentration of the samples used (Cuaresma Franco, 2011).

## 4.3. Results and discussion

### 4.3.1. Effect of the applied light-regime and the dynamically changing $O_2$ on algal growth

The specific growth rate of *Neochloris oleoabundans* under dynamic oxygen concentration and sub-saturated light conditions was determined. The experimental run can be divided in six different phases. Initially, the photobioreactor was inoculated with *Neochloris oleoabundans* and the cells were grown batch wise (Figure 1 – Phase 1) until the optical density was above 0.8 CU, reaching a biomass concentration of 1.05 ± 0.02 g $L^{-1}$. In Phase 2 the algal culture was diluted to the desired optical density with the addition of fresh culturing medium and allowed to adjust to continuous sub-saturating light conditions and constant partial oxygen pressure of 0.21 bar for 2 days. After this initial acclimation phase, the optical density was kept constant corresponding to a biomass concentration of 0.55 ± 0.02 g $L^{-1}$ and showed a specific growth rate of 1.14 ± 0.06 $day^{-1}$ (Table 1).

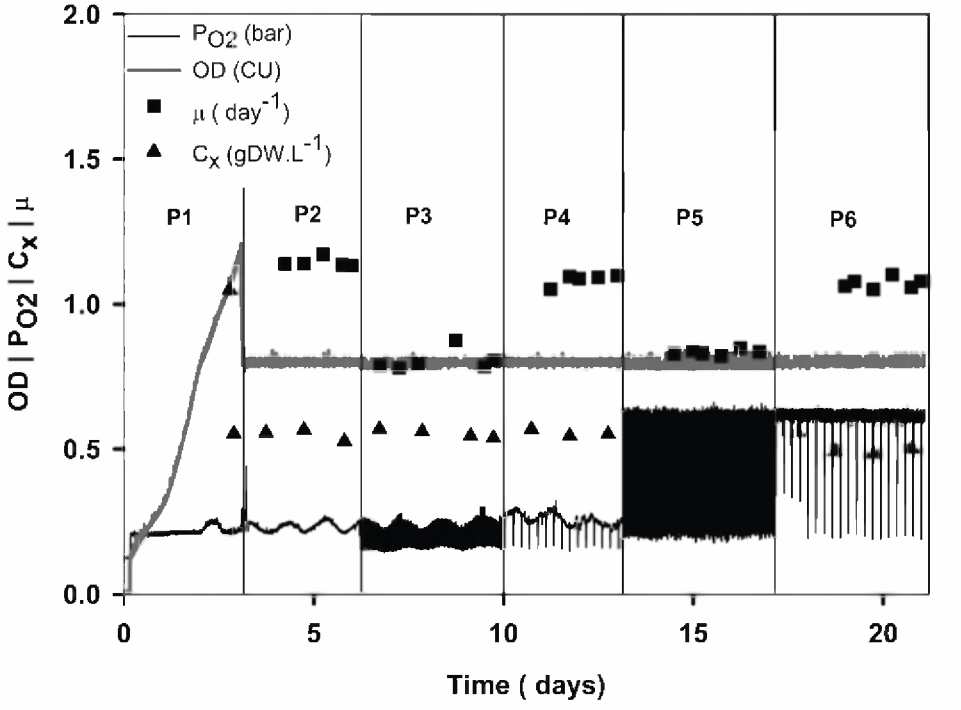


***Figure 1*** *- Graphical representation of partial oxygen pressure ($P_{O_2}$) in bar; optical density (OD) in CU; specific growth rate (μ) in day$^{-1}$; biomass concentration (Cx) in g L$^{-1}$ versus time in days. P#= Phase*

During this experiment a typical circadian (daily) rhythm in $O_2$ production can be observed (Figure 1). Circadian rhythms are endogenous biological programs that time different physiological processes to occur at optimal phases during the daily cycle, such as cell division, gene expression, etc. (Suzuki & Johnson, 2001). This circadian rhythm influences the determination of the specific growth rate and cell concentration, and therefore samples were taken at daily intervals.

In Phase 3 the oxygen concentration was controlled at $P_{O_2}$ = 0.21 bar, but now the light was turned on for 30 minutes and turned off during 6 minutes (light regime 30/6) to mimic the darkness during degassing. The biomass concentration was constant at 0.55 ± 0.01 g L$^{-1}$ and after 4 days the specific growth rate was determined to be 0.8 ± 0.16 day$^{-1}$, showing that the algal growth rate decreased

due to the light regime applied in the experiments. This measured specific growth rate was used as reference value for the specific growth rate measured during the phase in which the effect of dynamically changing oxygen concentration is applied at the same light regime of 30/6.

In Phase 4 the oxygen was controlled at a constant partial pressure of 0.21 bar and a light regime corresponding to 300 minutes lights on and 6 minutes lights off (300/6) was applied. The biomass concentration was again 0.55 ± 0.01 g $L^{-1}$ and the specific growth rate obtained after 3 days was 1.09 ± 0.05 $day^{-1}$ and thus only 4% lower than the growth rate at continuous light and about 1.4 times higher than at a light regime of 30/6. This specific growth rate served as reference for the final phase of the experiment when the algae growths were investigated when exposed to high oxygen levels for 300 minutes.

***Table 1*** *- Average biomass concentration, specific growth rate and biomass yield on photons of Neochloris oleoabundans during each phase of the experimental run*

| | $C_x$ ± Stdev (g $L^{-1}$) | μ ± Stdev ($day^{-1}$) | $Y_{x,ph}$ ± Stdev (g $mol\text{-}ph^{-1}$) |
|---|---|---|---|
| P1 – Batch | 1.05 ± 0.02 | N/A | N/A |
| P2 - Continuous light - $P_{O_2}$=0.21 bar | 0.55 ± 0.02 | 1.14 ± 0.06 | 1.15 ± 0.04 |
| P3 - Light regime 30/6 - $P_{O_2}$=0.21 bar | 0.55 ± 0.01 | 0.80 ± 0.16 | 0.97 ± 0.03 |
| P4 - Light regime 300/6 - $P_{O_2}$=0.21 bar | 0.55 ± 0.01 | 1.09 ± 0.05 | 1.12±0.02 |
| P5 - Light regime 30/6 - Dynamic $P_{O_2}$=0.21/0.63 bar | 0.49 ± 0.01 | 0.82 ± 0.04 | 0.88 ± 0.02 |
| P6 - Light regime 300/6 - Dynamic $P_{O_2}$=0.21/0.63 bar | 0.51 ± 0.03 | 1.07 ± 0.12 | 1.01 ± 0.07 |

During Phase 5 dynamic oxygen conditions were applied and the reactor was operated at a light regime of 30 minutes lights “On” and 6 minutes lights “Off”. The oxygen was allowed to build up from $P_{O_2}$ = 0.21 bar to $P_{O_2}$ = 0.63 bar for 30 minutes and then was forced to decrease to starting level during 6 minutes of degassing. At those conditions a specific growth rate of 0.82 ± 0.04 $day^{-1}$ (Table 1) was measured which does not differ from the specific growth rate of 0.80 ± 0.16

$day^{-1}$ measured in the reference experiment (Phase 3).This shows that the dynamic change in oxygen concentrations as such does not contribute to the decrease of the growth rate at the sub-saturating light conditions used.

The final phase (Phase 6) of this experiment was performed using an exposure time at high oxygen concentration of $P_{O_2}$ = 0.63 bar for 300 min, followed by 6 minutes for degassing and the light supply controlled using the 300/6 light regime. The specific growth of the algae measured was 1.07 ± 0.12 $day^{-1}$. With only a minor decrease in growth rate compared with the reference experiment (Phase 4), this result confirms once again that the decrease in the specific growth rate of *Neochloris oleoabundans* is not caused by the build-up of the oxygen concentration but that the light regime applied is far more important for the growth of the algae.

When comparing the specific growth rate at constant oxygen concentration ($P_{O_2}$=0.21 bar) and applying a light regime 30/6 (P3), with the one obtained at continuous light and constant $P_{O_2}$= 0.21 bar (P2), the dark period that algae were experiencing resulted in a decrease of the specific growth rate from 1.14 ± 0.06 $day^{-1}$ down to 0.80 ± 0.16 $day^{-1}$. At constant oxygen concentration ($P_{O_2}$= 0.21), light regime 300/6 (P4) and constant oxygen concentration ($P_{O_2}$= 0.21 bar), continuous light (P2) the decrease in growth rate was less profound. At a light regime 300/6 the algae were exposed to dark periods less often (compared with light regime 30/6) and the decrease in the specific growth rate was lower. The dynamic oxygen conditions for the both light regimes (30/6 and 300/6) (P5 and P6) did not give remarkable changes in the specific growth rate compared with the corresponding reference (P3 and P4) growth rates obtained at constant oxygen concentration ($P_{O_2}$= 0.21 bar).

### 4.3.2. Effect on biomass yield on photons

In Table 1 the biomass yield on photons, calculated based on the biomass production rate and light supply rate, is expressed as the amount of light energy that is converted into biomass per mol of photons supplied in the PAR range (g $mol\text{-}ph^{-1}$). Under dynamic oxygen concentrations a biomass yield on photons of 0.88 ± 0.02 g $mol\text{-}ph^{-1}$ (P5) and 1.01 ± 0.07 g $mol\text{-}ph^{-1}$ (P6) was calculated. The

maximum biomass yield on photons calculated in this experiment was 1.15 ± 0.04 g mol-ph$^{-1}$ (P2) under continuous light and constant oxygen concentration ($P_{O_2}$=0.21 bar). This value is higher than the value for *Neochloris oleoabundans* obtained by Pruvost et al. (2009). They reported a volumetric biomass productivity for *Neochloris oleoabundans* of 0.55 kg m$^{-3}$ day$^{-1}$ and when this value is combined with the provided data of incident light flux and the illuminated surface to volume ratio of the reactor used at continuous light conditions (light-limited growth), a biomass yield on photons of 0.71 g mol-ph$^{-1}$ is calculated. *Neochloris oleoabundans* exposed to dynamic oxygen concentrations and sub-saturating light conditions still exhibits similar high biomass yield on photons. In previous work on the effect of continuous oxygen concentrations ($P_{O_2}$=0.63 bar) on the algal growth of *Neochoris oleoabundans* at sub-saturating light intensities a biomass yield on photons of 1.07 ± 0.10 g mol-ph$^{-1}$ was obtained (Sousa et al., 2012). These results clearly show the effect of temporary exposure of the algae to dark regimes. Evaluating the results from P3 and P4 it is fair to say, that once the light exposure was increased by a factor 10 we would expect a larger increase in specific growth rate than the 1.4 times found. In addition, a similar biomass yield in photons would be expected during the whole experiment. But we have to take in consideration, that not all the light that falls on the photosynthetic antenna complexes is absorbed. A part of the light is absorbed and used for photosynthesis but another part just passes through the cells. Another factor to consider is the energy required for maintenance. All the processes needed for the algae to survive except growth, also require energy and during the darkness periods, energy is needed for respiration and storage of compounds (Vejrazka et al., 2011).

### 4.3.3. Chlorophyll and carotenoid content

To verify if application of different light regimes and dynamically changing oxygen concentration lead to additional photo-acclimation and photo-inhibition effects on the growth rate, the chlorophyll and carotenoid content of the algae was measured during the subsequent phases of the experiment (Figure 2).

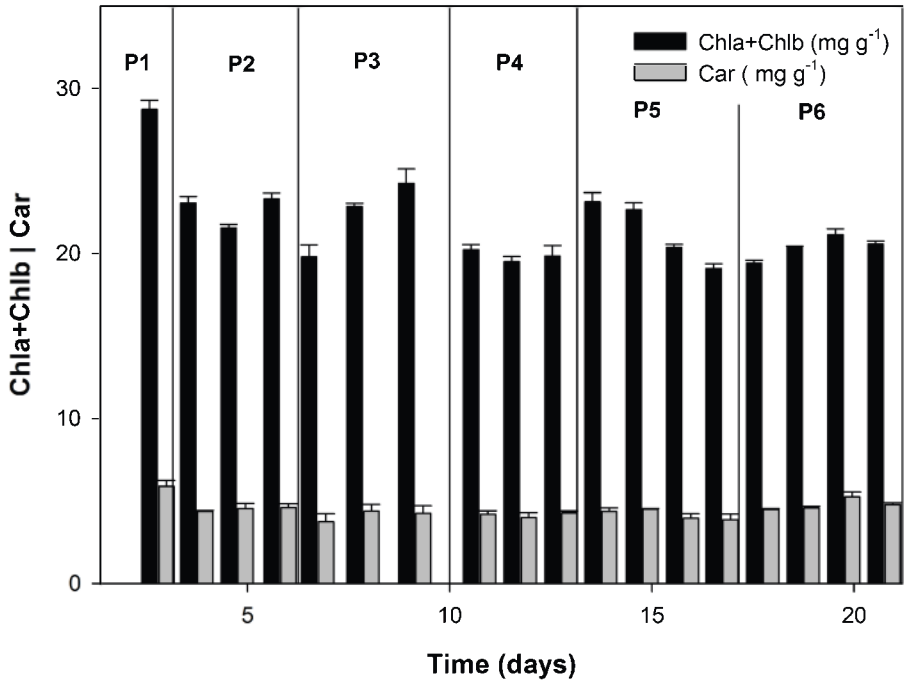


***Figure 2*** *- Total chlorophyll content and carotenoids in Neochloris oleoabundans at different phases in the experimental run. P= Phase (see Table 1 for the conditions at which the samples were taken)*

The chlorophyll content of the algae was followed during the whole experimental run and the cells show typical photo-acclimation effects. Chlorophyll content decreased when more light was available per cell and increased in case less light was provided The highest amount of chlorophyll per dry weight was measured in P1 (29 mg $g^{-1}$) at the end of the batch, when the biomass density inside the photobioreactor was relatively high and less light was available per cell. During turbidostat operation, the chlorophyll content was in general lower, as the biomass density was kept constant at relative low biomass concentration (Figure 1).The differences in chlorophyll content observed during the different turbidostat phases was not caused by the higher oxygen concentrations applied but due to the different exposure times of the algae to the light. The shortest (P3 and P5) periods of exposure resulted in higher chlorophyll concentrations and the longest exposures of time (P4 and P6) in lower concentrations. No differences can be observed when comparing the average chlorophyll content of the cells exposed to

the same light regime, but at low oxygen concentration (P3 and P4) and the chlorophyll content of the cells subjected to dynamically changing oxygen concentrations (P5 and P6).

Carotenoids are known as photoprotective pigments. Their content normally increases when the algae cells are exposed to high light intensities (Dubinsky & Stambler, 2009). The reason for that is their function to dissipate excess energy of exited chlorophyll and also eliminating ROS (Lawlor, 2001). The amount of carotenoids remained constant over the whole experiment. Neither the oxygen levels in the medium, nor the light regimes applied affected the carotenoid content, which indicates that additional photo-inhibition effects did not interfere at the light conditions used. In addition, the applied oxygen concentration did not lead to extra formation of this photoprotective pigment, demonstrating that oxygen as such does not induce photoinhibition.

### 4.3.4. Final remarks

In closed photobioreactor systems, oxygen accumulation leads to an increase of the $O_2/CO_2$ ratio in the medium, promoting the oxygenase activity of the enzyme and activating the photorespiratory pathway. Dissolved oxygen concentrations in photobioreactors can easily increase up to 4 times air saturation (Carvalho et al., 2006; Weissman et al., 1988). The oxygen build-up and accumulation is a particularly serious problem which ultimately results in decreases in specific growth rates and biomass productivities. Oxygen concentrations above 1.5 to 2 times air saturation inhibit algal growth (Ota et al., 2011; Raso et al., 2011; Ugwu et al., 2007). For work performed at outdoor conditions, the oxygen problem is taken into consideration by working at oxygen concentrations below inhibiting levels for the microalgal cultures. The most commonly used strategy to maintain the oxygen level below inhibiting levels is the implementation of degassers (Fernandez et al., 2001; Fuentes et al., 1999; Pirt et al., 1983; Richmond et al., 1993; Travieso et al., 2001). In a review of enclosed system designs and performances Carvalho et al. (2006) state that a universally optimum gas transfer device does not exist. In all the cases, an overall decrease in specific growth rates under oxygen accumulation was observed and/or expected. Based on that, a lower growth rate was also expected when dynamically changing oxygen

concentrations were applied, once the microalgae cultures were anyhow exposed to high oxygen concentrations for a period of time. The results obtained in this study however, showed no significant change in the specific growth rate caused by the high oxygen concentration. In addition, high oxygen concentrations did not lead to changes in pigmentation, which indicates that the cells did not activate protective mechanisms against photooxidative damage. The observation that the growth rate and the biomass yield on photons is not affected by the dynamically changing oxygen concentration could be explained by the presence of a carbon concentrating mechanism (CCM) in *Neochloris oleoabundans* that is activated at elevated oxygen concentrations in the medium. CCM's are considered responsible for minimizing photorespiration by increasing the dissolved inorganic carbon concentration in the cell via active transport of $CO_2$ and $HCO_3^-$ (Kaplan & Reinhold, 1999). This increase in $CO_2$ concentration helps the carboxylation reaction and inhibits the oxygenase activity of the Rubisco. Consequently increases photosynthesis and reduces photorespiration (Huertas et al., 2000).

## 4.4. Conclusions

The effect of dynamic oxygen concentrations and light/dark regime of light on growth of *Neochloris oleoabundans* was evaluated. Gradual increase of the oxygen concentration from $P_{O_2}$ = 0.21 bar up to 0.63 bar, followed by period of rapid degassing did not bring significant decrease in the specific growth rate. Furthermore even when the algae were exposed for 300 minutes at high oxygen concentration, there was no significant change in the specific growth rate of the algae *Neochloris oleoabundans.* Obtained results show that the residence time of the algae on the solar receiver could be increased up to 10 x without degassing. A significant decrease of the algae specific growth was observed when applying light regime of 30 minutes light "On" and 6 minutes lights "Off", proving that the exposure of the algae cells to dark periods in the degasser has a bigger negative impact than the exposure to high oxygen concentration as such. The results of this study clearly show that optimization of closed photobioreactors based on the observed algae physiology under dynamic oxygen concentration will result not only in a reduction of the number of the degassing units, but also by doing so it will contribute for keeping higher growth rate and higher biomass production

respectively. Reducing the amount of degassing units and increasing their degassing capacity, to minimize the time of darkness the algae are exposed to, will result in substantial savings in design and operation of plants for microalgae production.

## 4.5. Acknowledgements

This work was performed in the TTIW-cooperation framework of Wetsus, Centre of Excellence for Sustainable Water Technology (www.wetsus.nl). Wetsus is funded by the Dutch Ministry of Economic Affairs, the European Union Regional Development Fund, the Province of Fryslân, the City of Leeuwarden and the EZ/Kompas program of the "Samenwerkingsverband Noord-Nederland". The authors like to thank the participants of the research theme "Algae" for the discussions and their financial support.

## 4.6. References

Britton, G., Liaaen-Jensen, S., Pfander, H., Frank, H.A., Young, A.Y., Britton, G., Cogdell, R.J. 1999. The Photochemistry of Carotenoids. Kluwer Academic Publishers.

Carvalho, A.P., Meireles, L.A., Malcata, F.X. 2006. Microalgal reactors: A review of enclosed system designs and performances. Biotechnology Progress, 22(6), 1490-1506.

Cuaresma Franco, M. 2011. Cultivation of microalgae in a high irradiance area, PhD thesis, Wageningen University, the Netherlands.

Demmig-Adams, B., Adams, W.W. 2002. Antioxidants in Photosynthesis and Human Nutrition. Science, 298 (5601 ), 2149 - 2153

Dubinsky, Z., Stambler, N. 2009. Photoacclimation processes in phytoplankton: mechanisms, consequences, and applications. Aquatic Microbial Ecology, 56(2-3), 163-176.

Falkowski, P.G., Raven, J.A. 2007. Aquatic photosynthesis. 2nd ed. Princeton University Press, Princeton.

Fernandez, F.G.A., Sevilla, J.M.F., Perez, J.A.S., Grima, E.M., Chisti, M.Y. 2001. Airlift-driven external-loop tubular photobioreactors for outdoor production of microalgae: assesment of design and performance. Chemical Engineering Science, 56(8), 2721-2732.

Foyer, C.H., Bloom, A.J., Queval, G., Noctor, G. 2009. Photorespiratory metabolism: genes, mutants, energetics, and redox signaling. Annual Review of Plant Biology, 60(1), 455-484.

Fuentes, M.M.R., Sanchez, J.L.G., Sevilla, J.M.F., Fernandez, F.G.A., Sanchez-Perez, J.A., Grima, E.M. 1999. Outdoor continuous culture of *Porphyridium cruentum* in a tubular photobioreactor: quantitative analysis of the daily cyclic variation of culture parameters. Journal of Biotechnology, 70(1-3), 271-288.

Guillard, R.R.L., Ryther, J.H. 1962. Studies of marine planktonic diatoms. *I. Cyclotella nana hustedt*, and *Detonula confervagea (Cleve) gran*. Canadian Journal for Microbiology, 8, 229-239.

Huertas, I.E., Espie, G.S., Colman, B., Lubian, L.M. 2000. Light-dependent bicarbonate uptake and $CO_2$ efflux in the marine microalga *Nannochloropsis gaditana*. Planta, 211(1), 43-49.

Kaplan, A., Reinhold, L. 1999. $CO_2$ concentrating mechanisms in photosynthetic microorganisms. Annual Review of Plant Physiology and Plant Molecular Biology, 50, 539-+.

Kliphuis, A.M.J., Martens, D.E., Janssen, M., Wijffels, R.H. 2011. Effect of $O_2$:$CO_2$ ratio on the primary metabolism of *Chlamydomonas reinhardtii*. Biotechnology and Bioengineering, 108(10), 2390-2402.

Lamers, P.P., Janssen, M., De Vos, R.C.H., Bino, R.J., Wijffels, R.H. 2008. Exploring and exploiting carotenoid accumulation in *Dunaliella salina* for cell-factory applications. Trends in Biotechnology, 26(11), 631-638.

Lawlor, D.W. 2001. Photosynthesis. Bios Scientific Publishers, Oxford.

Miron, A.S., Gomez, A.C., Camacho, F.G., Grima, E.M., Chisti, Y. 1999. Comparative evaluation of compact photobioreactors for large-scale monoculture of microalgae. Journal of Biotechnology, 70(1-3), 249-270.

Norsker, N.-H., Barbosa, M.J., Vermuë, M.H., Wijffels, R.H. 2011. Microalgal production -- A close look at the economics. Biotechnology Advances, 29(1), 24-27.

Ota, M., Kato, Y., Watanabe, M., Sato, Y., Smith Jr, R.L., Rosello-Sastre, R., Posten, C., Inomata, H. 2011. Effects of nitrate and oxygen on photoautotrophic lipid production from *Chlorococcum littorale*. Bioresource Technology, 102(3), 3286-3292.

Pirt, S.J., Lee, Y.K., Walach, M.R., Watts Pirt, M., Balyuzi, H.H.M., Bazin, M.J. 1983. A tubular bioreactor for photosynthetic production of biomass from carbon dioxide: Design and performance. Journal of Chemical Technology and Biotechnology, 33B, 35-58.

Pruvost, J., Van Vooren, G., Cogne, G., Legrand, J. 2009. Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. Bioresource Technology, 100(23), 5988-5995.

Raso, S., van Genugten, B., Vermuë, M., Wijffels, R.H. 2011. Effect of oxygen concentration on the growth of *Nannochloropsis sp.* at low light intensity. Journal of Applied Phycology, 1-9.

Richmond, A., Boussiba, S., Vonshak, A., Kopel, R. 1993. A new tubular reactor for mass production of microalgae outdoors. Journal of Applied Phycology, 5(3), 327-332.

Sousa, C., de Winter, L., Janssen, M., Vermuë, M.H., Wijffels, R.H. 2012. Growth of the microalgae *Neochloris oleoabundans* at high partial oxygen pressures and sub-saturating light intensity. Bioresource Technology, 104(0), 565-570.

Suzuki, L., Johnson, C.H. 2001. Algae know the time of day: Circadian and photoperiodic programs. Journal of Phycology, 37, 933-942.

Travieso, L., Hall, D.O., Rao, K.K., Benitez, F., Sanchez, E., Borja, R. 2001. A helical tubular photobioreactor producing Spirulina in a semicontinuous mode. International Biodeterioration & Biodegradation, 47(3), 151-155.

Triantaphylidès, C., Havaux, M. 2009. Singlet oxygen in plants: production, detoxification and signaling. Trends in Plant Science, 14(4), 219-228.

Ugwu, C.U., Aoyagi, H., Uchiyama, H. 2007. Influence of irradiance, dissolved oxygen concentration, and temperature on the growth of *Chlorella sorokiniana*. Photosynthetica, 45(2), 309-311.

Ugwu, C.U., Aoyagi, H., Uchiyama, H. 2008. Photobioreactors for mass cultivation of algae. Bioresource Technology, 99(10), 4021-4028.

Vejrazka, C., Janssen, M., Streefland, M., Wijffels, R.H. 2011. Photosynthetic Efficiency of *Chlamydomonas reinhardtii* in Flashing Light. Biotechnology and Bioengineering, 108(12), 2905-2913.

Vilchez, C., Forjan, E., Cuaresma, M., Bedmar, F., Garbayo, I., Vega, J.M. 2011. Marine Carotenoids: Biological Functions and Commercial Applications. Marine Drugs, 9(3), 319-333.

Weissman, J.C., Goebel, R.P., Benemann, J.R. 1988. Photobioreactor design: Mixing, carbon utilization, and oxygen accumulation. Biotechnology and Bioengineering, 31, 336-344.

Wijffels, R.H., Barbosa, M.J., Eppink, M.H.M. 2010. Microalgae for the production of bulk chemicals and biofuels. Biofuels, Bioproducts and Biorefining, 4(3), 287-295.

# Chapter 5. EFFECT OF DYNAMIC OXYGEN CONCENTRATIONS ON THE GROWTH OF *NEOCHLORIS OLEOABUNDANS* AT HIGH LIGHT CONDITIONS

Claudia Sousa[1, 2], Marian H. Vermuë[2] and Rene H. Wijffels[2]

[1,] Wetsus, P.O. Box 1113, 8900 CC Leeuwarden, the Netherlands

[2,] Bioprocess Engineering, Wageningen University, P.O. Box 8129, 6700 EV, Wageningen, the Netherlands

**Abstract** - Dynamically changing oxygen concentrations experienced in closed photo-bioreactor system were simulated in CSTR at high light intensity. The effect of 10 times elongation of the residence time in the solar receiver was investigated. When the algae were exposed to constant oxygen concentration and constant high light the specific growth rate was 1.29 ± 0.08 $day^{-1}$. Using a light regime of 30 minutes light ON followed by 6 minutes lights OFF and degassing resulted in a specific growth rate of 0.84 ± 0.09 $day^{-1}$, elongation of the time (lights ON) to 300 minutes resulted in 1.18 ± 0.05 $day^{-1}$. When dynamically changing oxygen concentrations were applied, similar specific growth rates were obtained. These results indicate that algae do not experience the expected photo-oxidative inhibition caused by high oxygen concentration in combination with high light, as long as the oxygen is removed via regular degassing. The temporary exposure of the algae to the darkness in the degasser has more impact on the productivity.

**Key words:** tubular photobioreactors, dynamic oxygen, *Neochloris oleoabundans,* high light intensities

*Submitted for publication*

## 5.1. Introduction

High growth rates and high lipid content combined with the non-competitiveness with food, make microalgae one of the most attractive resource for biodiesel production (Gong & Jiang, 2011; Wijffels et al., 2010). To make large-scale outdoor production of microalgae for biodiesel economically feasible, design and development of photobioreactors for microalgal cultivation is subject of many studies that focus on maximization of the microalgae production (Norsker et al., 2011; Singh & Sharma, 2012; Wijffels & Barbosa, 2010). Tubular photobioreactors are presented as a cost efficient system with room for improvement (Norsker et al., 2011). These types of closed photobioreactors are already used as outdoor cultivation systems for microalgae (Molina et al., 2001; Pirt et al., 1983; Torzillo et al., 1993; Tredici & Zittelli, 1998) but a lot of energy is used for the $CO_2$ supply and to prevent $O_2$ accumulation (Norsker et al., 2011). Studies on the effect of oxygen on microalgae growth at constant oxygen concentrations and constant light intensities revealed that $O_2$ accumulation leads to a decrease in specific growth rate (Kliphuis et al., 2011; Raso et al., 2011; Sousa et al., 2012). In practice, however, algae cultured in tubular photobioreactor systems are not subjected to constant oxygen concentrations, but they experience an increasing oxygen gradient along the tube length and at the end of the tube the high oxygen level could lead to decreasing growth rate (Ugwu et al., 2008). Therefore at the end of the tube a degassing unit is placed, where the accumulated oxygen is removed to. To test if indeed, the accumulation of oxygen caused a decrease of the growth rate, Sousa et al (2013b) measured the *in-vivo* growth rate of *Neochloris oleoabundans* at dynamically changing oxygen concentrations ($P_{O_2}$=0.21/0.63 bar) and low light intensity (200 µmol $m^{-2}$ $s^{-1}$) followed by a short dark period of degassing to simulate the dynamically changing oxygen and light conditions experienced by algae in a tubular photobioreactor system. Surprisingly, no significant decrease in specific growth rate was found. At these low light intensities, the grow rate decreased only due to the effect of less light availability during the simulated dark periods in the degasser. This indicated that $O_2$ accumulation did not lead to additional photorespiration effects as long as the $O_2$ was removed frequently (Sousa et al., 2013b).

In outdoor conditions light conditions vary over the day and over the seasons. Moreover, especially during the start-up of the cultures when the biomass concentration is still low, the algae may also experience higher light intensities. At high light intensities, the combination of high $O_2$ and high light additional photoinhibition effects can be expected (Kliphuis et al., 2011; Raso et al., 2011; Sousa et al., 2012) due to the formation of oxygen radicals and other reactive oxygen species (ROS) (Murata et al., 2007) and formation of the highly reactive singlet oxygen via photoactivation (Triantaphylides et al., 2008). It is not clear however, how the algae will respond on the dynamically changing oxygen concentrations in a tubular photobioreactor system while the algae are exposed to high light intensities followed by short periods of darkness in the degasser. To simulate these cultivation conditions, a fully controlled Continuous Stirred Tank Reactor (CSTR) operated in turbidostat mode was used to culture *Neochloris oleoabundans* (UTEX1185) at high light intensity (500 µmol $m^{-2}$ $s^{-1}$) and the specific growth rate was measured. In addition, the time at which the algae were exposed to high partial oxygen pressures was extended to determine the effect of a ten times longer residence time at high light conditions on the specific growth rate of the algae. This experiment was included because in previous work at low light conditions the elongation of the time spent in the tubes, did not affect the specific growth rate. If extension of the time spend at high oxygen concentration and high light irradiance does not affect the growth rate, this would indicate that the period that cells spend in the dark degasser can be decreased considerably, leading to higher productivities.

## 5.2. Material and method

### 5.2.1. Culture and photobioreactor system

*Neochloris oleoabundans* (UTEX 1185) was pre-cultured in adapted f/2 medium (Guillard & Ryther, 1962) and used as inoculums in 3L bioreactor (Applikon Biotechnology, The Netherlands), operated in turbidostat mode. The reactor system and controlling apparatus is described by Sousa et al. (2012).

### 5.2.2. Light regime

Light was provided by two LED light panels (20x20 cm) (SL3500, Photon Systems Instruments, Czech Republic), which were set to supply an average incident light of ~500 µmol $m^{-2}$ $s^{-1}$ on the surface of the reactor walls. The light sources were connected to a Siemens PLC Relay (LOGO!) light controller to simulate the darkness period in the degasser of a tubular PBR system. Two different time regimes for the light were used, related with the oxygen conditions applied during the experiments. The first one was 30 minutes light "On" and 6 minutes light "Off" (30/6 regime). This regime was applied during dynamic oxygen conditions, when the algae were allowed to produce and accumulate oxygen from $P_{O_2}$=0.21 bar up to $P_{O_2}$=0.63 bar. After this phase a degassing phase was initiated. During the degassing phase the light controller switch to Lights "Off" regime allowing rapid degassing of the liquid volume back to starting oxygen levels of $P_{O_2}$=0.21 bar in 6 minutes.

The second time regime that was used, operated at 300 minutes light "On" and 6 minutes light "Off" (300/6 regime). This regime was applied during culturing the algae at high oxygen levels ($P_{O_2}$=0.63 bar) for 300 minutes. The degassing phase was performed at six minutes lights "Off".

### 5.2.3. Off-line analysis of samples

Triplicate samples of 5 ml were collected on a daily base for dry weight determination. The samples were diluted with 10 ml ammonium formate (0.5M) and filtered using pre-weighed glass fiber-filters (Whatman GF/F). An additional 40 ml of ammonium formate (0.5M) was used for washing the biomass on the filters. Filters with biomass were dried at 95 °C for 24 hours in aluminium trays, cooled in desiccator for 2 hours and weighed on a 5 digit analytical balance (ME235P-SD, Sartorius, Germany).

For chlorophyll and total carotenoid determination samples were collected on a daily base and centrifuged at 3750 rpm for 10 min. at 4 °C. The pellets were frozen at -80°C. The pigment extraction was done by addition of 5mL of 100% methanol to each tube. The tubes were then placed for 5 min. in ultrasonic bath (Sonorex Digitec, Bandelin). Afterwards the samples were incubated for 40 min at 60 °C and

for another 15 min at 0 °C and centrifuged once more at the same conditions. The supernatant was collected and the chlorophyll and carotenoid was determined by measuring the optical density at 470nm, 652 nm and 665 nm in a UV-visible spectrophotometer (UV_1650 PC, Schimadzu). Modified Arnon's equations were used to determine the chlorophyll and carotenoid content (Lichtenthaler, 1987). Chlorophyll and carotenoid content were expressed per gram of biomass, calculated based on the dry weight concentration of the samples used (Cuaresma et al., 2011).

## 5.3. Results and discussion

### 5.3.1. Effect of dynamic $O_2$ and light regime on algal growth

Figure 1 shows two typical bioreactor runs to determine the effects of constant and dynamically changing oxygen concentration while using different time regimes for oxygen build-up at high light conditions followed by a period of darkness during which the oxygen is removed.

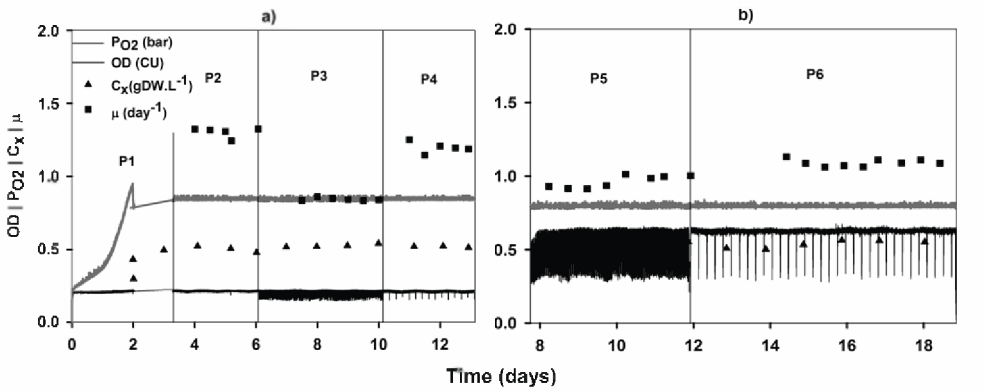


***Figure 1** - Graphical representation of partial oxygen pressure ($P_{O_2}$) in bar; optical density (OD) in CU; specific growth rate (μ) in day$^{-1}$; biomass concentration ($C_x$) in g L$^{-1}$ versus time in days on a typical experimental run.*
*(P2) Continuous Light - Constant $P_{O_2}$=0.21 bar; (P3) Light Regime 30/6 - Constant $P_{O_2}$=0.21 bar; (P4) Light Regime 300/6 - Constant $P_{O_2}$=0.21 bar; (P5) Light Regime 30/6 - Dynamic $P_{O_2}$=0.21/0.63 bar; (P6) Light Regime 300/6 - Dynamic $P_{O_2}$=0.21/0.63 bar.*

The experimental run represented in Figure 1 a) can be divided in 4 different phases. Initially, the photobioreactor was inoculated with *Neochloris oleoabundans* and the cells were grown batch wise (Figure 1a - Phase P1) until an optical density of 0.8 CU was reached, corresponding to a biomass concentration of 0.50 ± 0.02 g $L^{-1}$. In Phase P2 the algal culture was allowed to adjust to continuous high light conditions and a constant partial oxygen pressure of 0.21 bar. After an initial acclimation period of 2 days at a controlled optical density of 0.8 CU the specific growth rate was determined from the medium outflow of the photobioreactor and at these conditions the specific growth rate was found to be 1.29 ± 0.08 $day^{-1}$ (Figure 1a, Table 1). Phase P3 corresponds to a light regime 30 minutes light ON and 6 minutes light OFF at constant $P_{O_2}$ of 0.21 bar and Phase P4 300 minutes light ON and 6 minutes light OFF measured at the same constant $P_{O_2}$ of 0.21 bar.

In Figure 1 b), the measured data in the experiment with dynamically changing oxygen concentrations are shown. During Phase P5 dynamic oxygen conditions were applied and the reactor was operated at a light regime of 30 minutes lights "On" and 6 minutes lights "Off". The oxygen was allowed to build up from $P_{O_2}$=0.21 bar to $P_{O_2}$=0.63 bar for 30 minutes and then was forced to decrease to starting level during 6 minutes of degassing. At those conditions the specific growth rate was 0.97 ± 0.11 $day^{-1}$ (Table 1) and showed a higher specific growth rate than the one measured in the reference experiment (Phase P3).This shows that the dynamic change in oxygen concentrations as such, does not contribute to the decrease of the growth rate at the high light condition used. Phase P6 was performed using an exposure time at high oxygen concentration of $P_{O_2}$=0.63 bar for 300 min, followed by 6 minutes for degassing and the light supply controlled using the 300/6 light regime. The specific growth of the algae measured was 1.10 ± 0.1 $day^{-1}$. With no significant decrease in growth rate compared with the reference experiment (Phase P4), this result confirms once again that the decrease in the specific growth rate of *Neochloris oleoabundans* is not caused by the build-up of the oxygen concentration but that the light regime applied is more important for the growth of the algae.

***Table 1** - Average biomass concentration and specific growth rate of Neochloris oleoabundans during each phase of the experimental run*

| | $C_x$ ± Stdev ($g\ L^{-1}$) | μ ± Stdev ($day^{-1}$) |
|---|---|---|
| Batch phase (P1) | - | - |
| Continuous Light - Constant $P_{O_2}$=0.21 bar (P2) | 0.50 ± 0.02 | 1.29 ± 0.08 |
| Light Regime 30/6 - Constant $P_{O_2}$=0.21 bar (P3) | 0.53 ± 0.01 | 0.84 ± 0.09 |
| Light Regime 30/6 - Dynamic $P_{O_2}$=0.21/0.63 bar (P5) | 0.55 ± 0.02 | 0.97 ± 0.11 |
| Light Regime 300/6 - Constant $P_{O_2}$=0.21 bar (P4) | 0.52 ± 0.005 | 1.18 ± 0.14 |
| Light Regime 300/6 - Dynamic $P_{O_2}$=0.21/0.63 bar (P6) | 0.54 ± 0.03 | 1.10 ± 0.10 |

When comparing the specific growth rate at constant low oxygen concentration ($P_{O_2}$=0.21 bar) and applying a light regime 30/6 (P3), with the one obtained at continuous light and constant $P_{O_2}$=0.21 bar (P2), the dark period that algae were experiencing resulted in a decrease of the specific growth rate from 1.29 ± 0.08 $day^{-1}$ down to 0.84 ± 0.09 $day^{-1}$. At constant low oxygen concentration ($P_{O_2}$=0.21), light regime 300/6 (P4) and constant oxygen concentration ($P_{O_2}$=0.21 bar), continuous light (P2) the decrease in growth rate is much smaller. At a light regime 300/6 the algae were exposed to dark periods less often, compared with light regime 30/6 resulting in the observed decrease in the specific growth rate.

The dynamic oxygen conditions for the both light regimes (30/6 and 300/6) (P5 and P6) did not give remarkable changes in the specific growth rate compared with the corresponding references (P3 and P4) growth rates obtained at constant oxygen concentration ($P_{O_2}$=0.21 bar). When considering the productivity in closed photobioreactor systems these results show that the light input plays a far more important role than the oxygen build-up, as long as oxygen is removed at a regular basis.

## 5.3.2. Chlorophyll and carotenoid content

Being exposed to average high light conditions combined with high oxygen concentration the formation of ROS and singlet oxygen is bound to occur. Microalgae have developed photo adaptive and photo protective mechanisms to deal with such unfavorable photo-oxidative stress conditions, to protect their photosynthetic apparatus (Amaro et al., 2012; Dubinsky & Stambler, 2009). At elevated oxygen concentrations in the photobioreactor was thus expected to induce extra carotenoid production to protect the cells by quenching the electrons and act as anti-oxidant against the ROS formed. (Demmig-Adams & Adams, 2002)

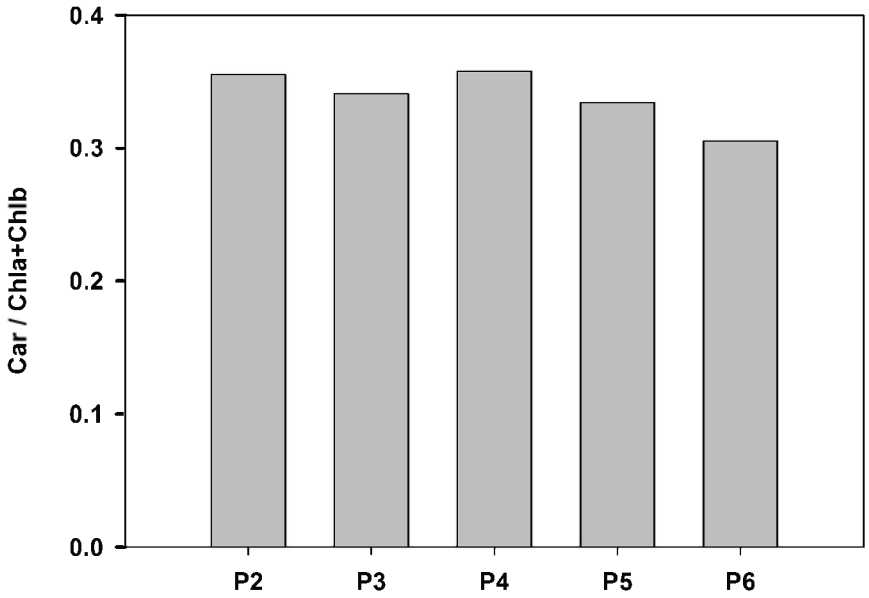


***Figure 2*** *- Ratio of total carotenoid and chlorophyll a+b content in Neochloris oleoabundans at the different phases.*
*P= Phase (see Figure1 for the description of the different phases)*

Figure 2 shows that the ratio of carotenoids and chlorophyll remained stable for all the phases. The expected change in pigmentation at dynamically changing

oxygen concentrations was not observed, indicating that the cells did not show any oxidative stress responses at the applied oxygen and light conditions.

## 5.4. Conclusions

The effect of dynamic oxygen concentrations and light/dark regime of high light on growth of *Neochloris oleoabundans* was evaluated. As in the previous work under low light intensity, the gradual increase of the partial oxygen concentration up to 0.63 bar, followed by period of rapid degassing did not bring significant decrease in the specific growth rate. Additionally when the algae were exposed for 300 minutes at high oxygen concentration, there was no significant change in the specific growth rate of the algae *Neochloris oleoabundans*. These results indicate that the algae do not experience the expected photo-oxidative inhibition caused by high oxygen concentration in combination with high light, as long as the oxygen is removed via regular degassing. But they also show that the temporary exposure to accumulating oxygen concentrations in the solar receiver has less impact on the growth rate than the residence time of the algae in the dark zone of the degasser. This indicates that the number of degassers in large-scale production of algae can be reduced without severe loss of biomass due to photorespiration and photo-oxidation. Moreover, decreasing the number of degasser will lead to increased productivity, as the algae spend relatively smaller time in the dark zone of the degasser.

## 5.5. Acknowledgements

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## 5.6. References

Amaro, H.M., Macedo, A.C., Malcata, F.X. 2012. Microalgae: An alternative as sustainable source of biofuels? Energy, 44(1), 158-166.

Demmig-Adams, B., Adams, W.W. 2002. Antioxidants in Photosynthesis and Human Nutrition. Science, 298 (5601 ), 2149 - 2153

Dubinsky, Z., Stambler, N. 2009. Photoacclimation processes in phytoplankton: mechanisms, consequences, and applications. Aquatic Microbial Ecology, 56(2-3), 163-176.

Gong, Y.M., Jiang, M.L. 2011. Biodiesel production with microalgae as feedstock: from strains to biodiesel. Biotechnology Letters, 33(7), 1269-1284.

Guillard, R.R.L., Ryther, J.H. 1962. Studies of marine planktonic diatoms. *I. Cyclotella nana hustedt*, and *Detonula confervagea (Cleve) gran*. Canadian Journal for Microbiology, 8, 229-239.

Kliphuis, A.M.J., Janssen, M., van den End, E.J., Martens, D.E., Wijffels, R.H. 2011. Light respiration in *Chlorella sorokiniana*. Journal of Applied Phycology, 1-13.

Lichtenthaler, H.K. 1987. [34] Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. in: Methods in Enzymology, (Ed.) R.D. Lester Packer, Vol. Volume 148, Academic Press, 350-382.

Molina, E., Fernandez, F.G.A., Chisti, M.Y. 2001. Tubular photobioreactor design for algal cultures. Journal of Biotechnology, 92, 113-131.

Norsker, N.H., Barbosa, M.J., Vermuë, M.H., Wijffels, R.H. 2011. Microalgal production - A close look at the economics. Biotechnology Advances, 29(1), 24-27.

Pirt, S.J., Lee, Y.K., Walach, M.R., Watts Pirt, M., Balyuzi, H.H.M., Bazin, M.J. 1983. A tubular bioreactor for photosynthetic production of biomass from carbon dioxide: Design and performance. Journal of Chemical Technology and Biotechnology, 33B, 35-58.

Raso, S., van Genugten, B., Vermuë, M., Wijffels, R.H. 2011. Effect of oxygen concentration on the growth of *Nannochloropsis sp.* at low light intensity. Journal of Applied Phycology, 1-9.

Singh, R.N., Sharma, S. 2012. Development of suitable photobioreactor for algae production – A review. Renewable and Sustainable Energy Reviews, 16(4), 2347-2353.

Sousa, C., de Winter, L., Janssen, M., Vermuë, M.H., Wijffels, R.H. 2012. Growth of the microalgae *Neochloris oleoabundans* at high partial oxygen pressures and sub-saturating light intensity. Bioresource Technology, 104(0), 565-570.

Sousa, C., Compadre, A., Vermuë, M.H., Wijffels, R.H. 2013a. Effect of oxygen at low and high light intensities on the growth of *Neochloris oleoabundans*. Algal Research, 2(2), 122-126.

Sousa, C., Valev, D., Vermuë, M.H., Wijffels, R.H. 2013b. Effect of Dynamic Oxygen Concentrations the growth of Neocholoris oleoabundans. Accepted for publication.

Torzillo, G., Carlozzi, P., Pushparaj, B., Montaini, E., Materassi, R. 1993. A two-plane tubular photobioreactor for outdoor culture of *Spirulina*. Biotechnology and Bioengineering, 42, 891-898.

Tredici, M.R., Zittelli, G.C. 1998. Efficiency of sunlight utilization: Tubular versus flat photobioreactors. Biotechnology and Bioengineering, 57(2), 187-197.

Wijffels, R.H., Barbosa, M.J. 2010. An outlook on microalgal biofuels. Science, 329(5993), 796-799.

Wijffels, R.H., Barbosa, M.J., Eppink, M.H.M. 2010. Microalgae for the production of bulk chemicals and biofuels. Biofuels, Bioproducts and Biorefining, 4(3), 287-295.

# Chapter 6. OXYGEN PRODUCTION IN PHOTOBIOREACTORS A LOOK TO THE ECONOMICS

Claudia Sousa [1, 2], Niels-Henrik Norsker[2, 3], Maria Barbosa[3, 4], Marian Vermuë [2,3], Rene H. Wijffels[2, 3]

[1] Wetsus, P.O. Box 1113, 8900 CC Leeuwarden, the Netherlands

[2] Bioprocess Engineering, Wageningen University, P.O. Box 8129, 6700 EV, Wageningen, the Netherlands

[3] Wageningen UR, AlgaePARC, www.AlgaePARC.com

[4] Food and Biobased Research, Wageningen UR, Bornse Weilanden 9, 6708 WG, Wageningen, the Netherlands

## 6.1. Introduction

Naturally oil-rich microalgae like *Neochloris oleoabundans* form an attractive renewable source for biofuel production. The outdoor large-scale production is technically feasible but still faces major challenges concerning the economic feasibility and the energy balance (Cheng & Timilsina, 2011; Norsker et al., 2011; Stephens et al., 2010a; Stephens et al., 2010b). .

For current outdoor production two major algae cultivation systems can be distinguished; open and closed systems. Open raceway ponds are the most used systems worldwide, because they are cheap in investments and operation costs. In addition, the energy balance for biomass production in these systems is positive (Norsker et al., 2011). The major drawback is that they are vulnerable for contamination and can only be used for production of fast growing algae or algae species that can grow under extreme conditions, like high salt or high pH, that prevent invasions by contaminants (Amaro et al., 2012; Pulz, 2001). Another disadvantage is that they operate at low biomass densities which make harvesting of the cells energy demanding and costly (Salim et al., 2012).

In closed photobioreactor systems (PBR) higher biomass densities can be achieved with smaller risk for contamination, but so far, no positive energy balance is achieved in these systems and the costs for production are still too high

(Dismukes et al., 2008; Norsker et al., 2011; Wijffels & Barbosa, 2010; Wijffels et al., 2010). Especially the high energy input required for mixing still forms a major bottleneck (Norsker et al., 2011). The mixing in closed PBR's is needed to provide the algae with sufficient light and carbon dioxide and to remove the photosynthetically produced oxygen. If not removed, the accumulating oxygen will inhibit the growth of the algae via photorespiration and will cause photo-oxidative damage as a result of photoinhibition, especially at high light intensity. There are indications, however, that the energy needed for mixing can be reduced considerably. (Norsker et al., 2011). Sousa et al. (2012) proved that addition of extra bicarbonate to the medium is a good method to overcome photorespiration and by doing so the algae can withstand high oxygen concentrations at low light intensities (Norsker et al., 2011; Sousa et al., 2012). This implies that less energy will be needed for degassing to remove the surplus of oxygen.

It was also found that algae can in fact withstand longer exposure to elevated oxygen concentrations, as long as the oxygen is frequently removed (Sousa et al. 2013b). This means that the energy needed for degassing can be further reduced. Here, an overview of the above-mentioned studies on effects of oxygen on the algal growth and the proposed methods to reduce the energy input for removal of the oxygen will be provided. The effects of implementing the proposed methods on the overall energy requirements and costs will be calculated, to verify if indeed these methods will contribute to a positive energy balance and a considerable reduction of the algal biomass cost.

### 6.1.1. Effects of oxygen on microalgal growth

In closed photobioreactors the oxygen produced during photosynthesis accumulates to high concentrations and induces processes like photoinhibition and photorespiration, both leading to a decrease of the yield on light of the microalgae (Torzillo et al., 1998).

### 6.1.1.1. Photorespiration

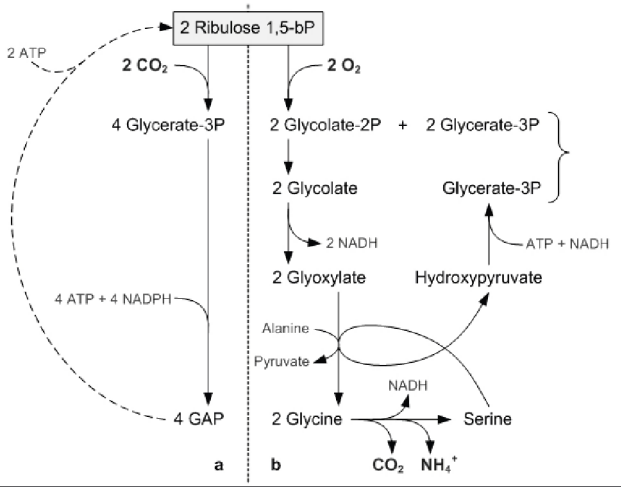


***Figure 1** - Simplified scheme of photosynthetic (a) and photorespiratory pathways (b) adapted from the metabolic network described by Kliphuis et al. (2011).*

Figure 1 shows the biochemical reactions involved in the photorespiratory pathway. Instead of two molecules of 3-phosphoglycerate (3-PGA), the reaction of ribulose bi-phosphate with $O_2$ yields one molecule of 3-PGA and one molecule of 2-phosphoglycolate (2-PG). The full photorespiratory cycle serves as a carbon recovery system converting part of the 2-PG to 3-PGA that can re-enter the reductive cycle. After 2-PG is dephosphorylated and the formed glycolate is converted into glycine, glycine is decarboxylated and deaminated for further serine synthesis and glycerate formation. The transport of glycerate and its phosphorylation to 3-PGA completes the photorespiratory pathway (Foyer et al., 2009; Tchernov et al., 2008; Tural & Moroney, 2005; Wingler et al., 2000). During photorespiration, $CO_2$ and ammonium ($NH_4^+$) are lost and their re-fixation requires additional ATP and NADPH. This means that less energy is available for growth and the biomass yield on light energy will decrease when photorespiration occurs (Kliphuis et al., 2011). The photorespiratory pathway thus has an influence on the photosynthetic yield which can be defined as the amount of $CO_2$ fixed per amount of light energy absorbed and, as such, will directly influence the productivity of microalgae cultures.

At sub-saturating light intensities the growth rate of microalgae decreases due to photorespiration (Figure 1). If the ratio between $O_2$ and $CO_2$ in the medium increases, the oxygenase activity of the enzyme Rubisco associated with respiration increases while its carboxylase activity associated to photosynthesis ceases. This photorespiration effect was indeed found for *Neochloris oleoabundans* cultured at sub-saturating light intensity upon increasing the oxygen concentration in the medium from 0.21 to 0.84 partial pressure (Sousa et al., 2012). To overcome the inhibitory effect of oxygen, the $CO_2$ concentration was increased by the addition of extra $NaHCO_3$ to the culture medium. Once the bicarbonate ($NaHCO_3$) concentration and the corresponding $CO_2$ concentration

were increased, the specific growth rate of the algae augmented again. This shows that the negative effect oxygen can be overcome by restoring the $O_2/CO_2$ ratio by an increase of the carbon dioxide partial pressure. By increasing the $NaHCO_3$ content in the medium, the oxygen is thus allowed to accumulate until partial pressure of 0.84 bar is reached, without compromising on productivity. Addition of extra $NaHCO_3$ could therefore reduce the energy needed for removal of the oxygen by degassing.

#### 6.1.1.2. Photoinhibition and photo-oxidative damage

At high and over-saturating light intensities additional photoinhibition effects were expected. At those conditions the formation of oxygen radicals and other reactive oxygen species (ROS) such as $H_2O_2$ happens (Murata et al., 2007). In addition, highly reactive singlet oxygen is formed via photo-activation (Triantaphylides et al., 2008) and causes photo-oxidative damage, resulting in a loss of photosynthetic activity and death of cells. The specific growth rate of *Neochloris oleobundans* cultured at near-saturating light conditions indeed dramatically decreased from 1.36 $day^{-1}$ at constant $P_{O_2}$ of 0.21 to 0.68 $day^{-1}$ at $P_{O_2}$ of 0.84. Contrary to what happened at sub-saturating light intensities, an increase of the $P_{CO_2}$ from 0.007 to 0.02 bar at $P_{O_2}$ of 0.84 bar did not have any positive effect on the overall growth of the algae. At these high light conditions, photoinhibition seem to dominate the photorespiration effects and the overall inhibitory effect of oxygen could not be overcome by addition of extra carbon dioxide.

One should realize that microalgae cultured in closed photobioreactors at high cell densities mainly encounter low light conditions. In particular in vertical stacked tubular photobioreactors or in vertical flat-panel reactors, the light is diluted. When the algal biomass density is high enough to prevent light conditions that evoke additional, photo-inhibition effects, the inhibitory effect of oxygen can be overcome by extra $NaCO_3$ addition to the medium. During the start-up of a culture, when the culture is still diluted, however, photo-oxidative damage is bound to occur and addition of extra $NaHCO_3$ will not help to reduce the inhibitory effects.

### 6.1.2. Effects of (dynamic) accumulating oxygen in closed photobioreactors

In closed tubular photobioreactor (PBR) the algae do not experience a constant oxygen partial pressure but they are subject to changing oxygen concentrations. The algae experience an increase in oxygen concentration along the length of the tubes, and this may induce reduction of the growth rate along the tubes (Ugwu et al., 2008). In addition, a degassing unit is placed at the end of the tube to remove the accumulated oxygen while which the algae are deprived from the light. This dynamic change in oxygen concentration in the tubes and the light conditions encountered during 6 minutes degassing were simulated in a fully controlled closed photobioreactor and the growth rate of *Neochloris oleobundans* at turbidostat conditions was measured. This growth rate was compared with the growth rate of the algae that were subjected to a ten times longer exposure time (300 minutes) to high oxygen concentrations. Surprisingly, the algae did not suffer from the extension of the residence time at high oxygen concentrations. The specific growth rate was not affected by the dynamically changing oxygen concentration, but only by the frequent exposure to dark periods encountered during degassing. A significant decrease of the algae specific growth was observed when applying a light regime of 30 minutes light “On” and 6 minutes lights “Off”.

The results of this study show that it is possible to increase the residence time in the solar receiver considerably. This can be done by decreasing the velocity in the tubes and by reducing the number of degassing units. Decreasing the velocity in the tubes will result in substantial energy and costs savings in design and operation of plants for microalgae production while reducing the amount of degassing units to minimize the time of darkness to which the algae are exposed, may result in higher productivity The turbulence created by the velocity is important to avoid the risk of algae deposition in the tube walls which can lead to biofouling. When reducing the velocity, the cost will be reduced but one should be aware that the risk of biofouling will increase.

## 6.2. Reducing the costs for degassing will reduce the overall costs

The presented studies indicate that the energy requirements for removing oxygen by degassing in closed photobioreactors can be substantially reduced. At constant sub-saturating light intensities it is possible to operate at oxygen concentration of 4 times air saturation by restoring the $CO_2/O_2$ ratio through increasing $P_{CO_2}$. Under dynamic oxygen concentrations, the inhibitory effect of oxygen on the specific growth rate of *Neochloris oleoabundans* was not found. Moreover; the residence time of the algae at high oxygen concentration could be increased up to 10 times without scrutinizing the growth rate. In fact, the exposure of the algae cells to dark periods in the degasser had a bigger negative impact than the exposure to high oxygen concentration as such.

### 6.2.1. Biomass productivity costs – base case

From the analysis of the above mentioned studies, it was concluded that the energy input for mixing and degassing in tubular photobioreactor systems could considerably be reduced. The effects of reducing the energy input on the overall energy balance as well as on the overall production costs were evaluated using the economic model developed by Norsker et al. (2011). The model was developed to calculate the energy and costs associated to microalgal biomass production in the Netherlands for three different systems at 100 ha scale. One of the systems was the horizontal tubular photobioreactor. This analysis resulted in a cost price of 4.15 € per Kg of biomass, and a negative net energy balance (25.5 MJ $kgDW^{-1}$) for production of algae biomass in these systems. All calculations were based on the assumption that the photosynthetic efficiency in the tubular system was 3% resulting in a areal productivity of 41.41 ton $ha^{-1}$ $yr^{-1}$ (Norsker et al., 2011). This value will be used as our base case.

### 6.2.2. Effect of increasing the $CO_2/O_2$ on biomass production costs

In the model the areal productivity was used as input parameter. The areal productivity was calculated using the measured biomass yield on light $Y_{x,ph}$ (g $mol\text{-}ph^{-1}$) that was measured in the previous studies (Sousa et al., 2012), combined with the yearly solar input of light in the Netherlands. The European Database of

Daylight and Solar Radiation reports a total solar radiation of $3.62x10^{13}$ J $ha^{-1}year^{-1}$ in the Netherlands and 42.3% of this solar irradiance can be used for photosynthesis (PAR, 400-700 nm wavelength), resulting in an average irradiance of $1.53x10^{13}$ J $ha^{-1}$ $year^{-1}$ on PAR light. Assuming an average wavelength of 550 nm, the average energy content of one mole of photons in the PAR range of light is $2.18x10^{5}$ J $mol\text{-}ph^{-1}$. This means an average yearly photon flux of about $7.02x10^{7}$ moles of PAR photons per ha. In our lab-scale experiments at sub-saturating light conditions and a partial oxygen pressure of 0.21 bar, a biomass yield $Y_{x,ph}$ of 1.04 g $mol\text{-}ph^{-1}$ was found (Sousa et al., 2012). Combined with the average yearly photon flux of 7.02 x $10^{7}$ moles of PAR photons per ha, this can be translated in an aerial productivity of 73 $ton.ha^{-1}.year^{-1}$. It is opportune to mention that the specific growth rates reported by Sousa et al. (2012) are comparable with the growth rates found by Pruvost et al. (2009). The biomass yields measured by Sousa et al. (2012) were nevertheless higher than those found by Pruvost et al. (2009). This shows that *N. oleoabundans* exhibits high biomass yields when grown on a marine salt water medium or on a freshwater BBM medium as used by Pruvost and coworkers. This outcome is not surprising since *N. oleoabundans* (UTEX 1185) has been isolated from an arid soil (Guiry, 2011). It is then necessary to take this in consideration when looking at the results of this study. The areal productivities calculated from the measured biomass yields greatly exceed the value used in the base case. The values for the areal productivities were substituted in the economic model and the biomass production costs and energy were calculated for an algal production facility with 100 hectares of tubular photobioreactors. It is necessary to take into account the measured biomass yield on light energy. The biomass yield on light energy measured at low partial oxygen pressure was 1.04 g $mol\text{-}ph^{-1}$ ($P_{O_2}$=0.21 bar | $P_{CO_2}$=0.007 bar) but dropped to 0.73 g $mol\text{-}ph^{-1}$ at high partial oxygen pressure ($P_{O_2}$=0.84 bar | $P_{CO_2}$=0.007 bar). Upon addition of $NaHCO_3$ it increased again to 0.92 $g.mol\text{-}ph^{-1}$ ($P_{O_2}$=0.84 bar | $P_{CO_2}$=0.02 bar) resulting in an areal productivity of 65 $ton.ha^{-1}yr^{-1}$. To achieve the productivity of 65 ton $ha^{-1}$ $yr^{-1}$ under a partial oxygen pressure of 0.84 bar (400% air saturation) the $CO_2$ partial pressure was increased from 0.007 bar to 0.02 bar by increasing the $NaHCO_3$ concentration in the medium (Sousa et al., 2012). The additional costs for the extra $CO_2$ should be taken into account when determining the overall costs. The

overall biomass production cost using 3 times more carbon dioxide but working at $P_{O_2}$=0.84 bar with *Neochloris oleoabundans* with a biomass yield on light energy under this circumstances of 0.92 g mol-ph$^{-1}$ is 3.60 € per kg. This is 13% less than calculated for the base case (4.15 € per kg). The net energy balance in these circumstances improved but is still negative (-6.73 MJ kgDW$^{-1}$).

### 6.2.3. Biomass productivity costs – increase the length of the tubes / decrease the velocity in the tubes.

In the lab-scale experiments in which we mimicked the dynamically changing oxygen concentration that the algae experience in the tubular system, we observed that the growth rate was hardly affected and that the algae are able to withstand 10 times longer residence times in the tubes at elevated oxygen concentrations. The residence time in the tube can be increased by lowering the velocity or by increasing the length of the tubes. Both methods will lead to a decrease in the amount of energy needed for mixing and degassing and in the total costs for algae production. Decreasing the velocity in the tubes will increase the time which the algae are in the solar receiver and will allow the oxygen to build up to higher values. Increasing the length of the tubes will as well increase the time in the solar receiver and allow the oxygen to build-up to high concentrations. Taking in consideration that this will not affect the growth of *Neochloris oleoabundans* as seen by the work on dynamic oxygen concentrations we calculated the biomass production cost under these circumstances. When increasing the length of the tubes while keeping the same velocity in the tubes, the biomass production cost hardly changed. The biomass production cost only decreased from 4.15 € per kg to 4.12€ per kg. Although Sousa et al (2013b) state that a 10x increase of the time to which the algae are exposed to sun is possible, the velocity in the tubes was only decreased from 0.5 m s$^{-1}$ to 0.25 m s$^{-1}$ as a too high reduction of the velocity might result in problems such as biofilm formation on the reactor walls. Decreasing the velocity by a factor of two results in a decrease on the biomass production cost of 4.15 € to 2.84 € per kg. This 32 % reduction on the cost of biomass production is mainly due to the required amount of circulation pumps and decreased energy requirement. In the base case analysis a negative net energy balance (-25.5 MJ kgDW$^{-1}$) was found. In the present case

the decrease of the velocity implicates a drastic reduction of the energy required for recirculation resulting in a positive net energy balance (+4.2 MJ $kgDW^{-1}$).

The evaluation of the dynamic oxygen concentrations in an outdoor pilot scale photobioreactor to validate the results found at lab-scale would be an interesting follow up to this research. It would be interesting to investigate to which extent the velocity in the tubes can be decreased to maximize the energy balance without excessive problems with biofouling. In the experiments at dynamic oxygen concentrations, the dark periods at which the algae were exposed were linked to the decrease of the specific growth rate. It would be interesting to study the effect on the energy balance of a combination of a decrease of the velocity in the tubes and of an increase in the degassing capacity of the degasser to reduce the dark time at which the algae are exposed.

## 6.3. Conclusions

The two methods adopted to overcome the negative effect of oxygen in microalgal cultures did result in a decrease in biomass production costs. At constant oxygen concentration, with expense of using more carbon dioxide the biomass production cost were reduced by 13 % and did improve the net energy balance, although it is still negative.

Under dynamic oxygen concentration the velocity can be decreased from 0.5 m $s^{-1}$ to 0.25 m $s^{-1}$ which results in a 32% saving on the biomass production cost but more important, it will result in a positive energy balance for tubular photobioreactors. This evaluation was done without taking in consideration methods to avoid biofilm deposition on the photobioreactors walls, what would be left here as a suggestion for further research. Evaluation of the dynamic oxygen concentrations in an outdoor pilot scale photobioreactor in order to validate the results found at lab-scale would be an interesting follow up to this research.

## 6.4. References

Amaro, H.M., Macedo, A.C., Malcata, F.X. 2012. Microalgae: An alternative as sustainable source of biofuels? Energy, 44(1), 158-166.

Cheng, J.J., Timilsina, G.R. 2011. Status and barriers of advanced biofuel technologies: A review. Renewable Energy, 36(12), 3541-3549.

Dismukes, G.C., Carrieri, D., Bennette, N., Ananyev, G.M., Posewitz, M.C. 2008. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Current Opinion in Biotechnology, 19(3), 235-240.

Foyer, C.H., Bloom, A.J., Queval, G., Noctor, G. 2009. Photorespiratory metabolism: genes, mutants, energetics, and redox signaling. Annual Review of Plant Biology, 60(1), 455-484.

Guiry, W., 2011. In Guiry, M.D. and Guiry, G.M. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org; searched on 10 October 2011.

Kliphuis, A.M.J., Martens, D.E., Janssen, M., Wijffels, R.H. 2011. Effect of $O_2$:$CO_2$ ratio on the primary metabolism of *Chlamydomonas reinhardtii*. Biotechnology and Bioengineering, 108(10), 2390-2402.

Murata, N., Takahashi, S., Nishiyama, Y., Allakhverdiev, S.I. 2007. Photoinhibition of photosystem II under environmental stress. Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1767(6), 414-421.

Norsker, N.-H., Barbosa, M.J., Vermuë, M.H., Wijffels, R.H. 2011. Microalgal production -- A close look at the economics. Biotechnology Advances, 29(1), 24-27.

Pruvost, J., Van Vooren, G., Cogne, G., Legrand, J. 2009. Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. Bioresource Technology, 100(23), 5988-5995.

Pulz, O. 2001. Photobioreactors: production systems for phototrophic microorganisms. Applied Microbiology and Biotechnology, 57, 287-293.

Salim, S., Vermuë, M.H., Wijffels, R.H. 2012. Ratio between autoflocculating and target microalgae affects the energy-efficient harvesting by bio-flocculation. Bioresource Technology, 118, 49-55.

Sousa, C., de Winter, L., Janssen, M., Vermuë, M.H., Wijffels, R.H. 2012. Growth of the microalgae *Neochloris oleoabundans* at high partial oxygen pressures and sub-saturating light intensity. Bioresource Technology, 104(0), 565-570.

Sousa, C., Compadre, A., Vermuë, M.H., Wijffels, R.H. 2013a. Effect of oxygen at low and high light intensities on the growth of *Neochloris oleoabundans*. Algal Research, 2(2), 122-126.

Sousa, C., Valev, D., Vermuë, M.H., Wijffels, R.H. 2013b. Effect of Dynamic Oxygen Concentrations the growth of Neocholoris oleoabundans. Accepted for publication.

Stephens, E., Ross, I.L., King, Z., Mussgnug, J.H., Kruse, O., Posten, C., Borowitzka, M.A., Hankamer, B. 2010a. An economic and technical evaluation of microalgal biofuels. Nat Biotech, 28(2), 126-128.

Stephens, E., Ross, I.L., Mussgnug, J.H., Wagner, L.D., Borowitzka, M.A., Posten, C., Kruse, O., Hankamer, B. 2010b. Future prospects of microalgal biofuel production systems. Trends in Plant Science, 15(10), 554-564.

Tchernov, D., Livne, A., Kaplan, A., Sukenik, A. 2008. The kinetic properties of ribulose-1,5-bisphosphate carboxylase/oxygenase may explain the high apparent photosynthetic affinity of *Nannochloropsis sp*. to ambient inorganic carbon. Israel Journal of Plant Sciences, 56(1-2), 37-44.

Torzillo, G., Bernardini, P., Masojidek, J. 1998. On-line monitoring of chlorophyll fluorescence to assess the extent of photoinhibition of photosynthesis induced by high oxygen concentration and low temperature and its effect on the productivity of outdoor cultures of *Spirulina platensis* (cyanobacteria). Journal of Phycology, 34 504 - 510

Triantaphylides, C., Krischke, M., Hoeberichts, F.A., Ksas, B., Gresser, G., Havaux, M., Van Breusegem, F., Mueller, M.J. 2008. Singlet oxygen is the major reactive oxygen species involved in photooxidative damage to plants. Plant Physiology, 148(2), 960-968.

Tural, B., Moroney, J.V. 2005. Regulation of the expression of photorespiratory genes in *Chlamydomonas reinhardtii*. Canadian Journal of Botany, 83(7), 810-819.

Ugwu, C.U., Aoyagi, H., Uchiyama, H. 2008. Photobioreactors for mass cultivation of algae. Bioresource Technology, 99(10), 4021-4028.

Wijffels, R.H., Barbosa, M.J. 2010. An outlook on microalgal biofuels. Science, 329(5993), 796-799.

Wijffels, R.H., Barbosa, M.J., Eppink, M.H.M. 2010. Microalgae for the production of bulk chemicals and biofuels. Biofuels, Bioproducts and Biorefining, 4(3), 287-295.

Wingler, A., Lea, P.J., Quick, W.P., Leegood, R.C. 2000. Photorespiration: metabolic pathways and their role in stress protection. Philosophical Transactions of the Royal Society B-Biological Sciences, 355(1402), 1517-1529.

# SUMMARY

Phototropic microalgae are regarded as a promising feedstock for sustainable biodiesel production, as microalgae can use natural sunlight as light source and are able to utilize $CO_2$ from flue gases and nutrients (P, N) from waste streams. To make large-scale outdoor microalgae production in closed photobioreactors economically feasible and sustainable, the costs for mixing and degassing should be reduced and the overall energy balance should become positive. The mixing is needed for effective and efficient supply of light, provision of carbon dioxide and degassing is needed for the removal of photosynthetically generated oxygen.

This thesis focused on the effect of the accumulation of oxygen on the growth of the oleaginous microalga *Neochloris oleoabundans* at different light intensities. These studies show at what concentrations oxygen becomes toxic for the algae at the different light conditions encountered during outdoor cultivation. This reveals when the oxygen should be removed from the photobioreactor and thus the need for degassing.

The different oxygen levels reached in outdoor tubular photobioreactors have been imposed on *Neochloris oleoabundans* while cultured in a fully controlled stirred tank reactor, operated in turbidostat. Under continuous illumination of 200 µmol $m^{-2}$ $s^{-1}$ (sub-saturating light intensity) the growth rate of *Neochloris oleoabundans* at the three oxygen partial pressures ($P_{O_2}$= 0.24; 0.63; 0.84) was 1.38; 1.36 and 1.06 $day^{-1}$ respectively was measured. At the oxygen partial pressure of 0.84 bar the carbon dioxide partial pressure ($P_{CO_2}$) was increased from 0.007 to 0.02 bar to see if the inhibiting effect of photorespiration could be overcome. Indeed, the addition of carbon dioxide resulted in an increase of the growth rate from 1.06 to 1.36 $day^{-1}$. The increase of specific growth rate confirmed that photorespiration was taking place and that this negative effect can be overcome by restoring the $CO_2/O_2$ ratio. In **Chapter 3** the effect of partial oxygen pressure on growth of *Neochloris oleoabundans* was studied at near-saturating light intensity in a fully-controlled photobioreactor. At the partial oxygen pressures tested ($P_{O_2}$= 0.24; 0.42; 0.63; 0.84 bar), the specific growth rate was 1.36; 1.16; 0.93 and 0.68 $day^{-1}$, respectively. An increase of the $P_{CO_2}$ from

0.007 to 0.02 bar at $P_{O_2}$ of 0.84 bar at these higher light intensities, however, did not show any positive effect on the overall growth of the algae, contrary to what happens at sub-saturating light intensities. These results indicate that at saturating light intensity the inhibitory effect of oxygen by photorespiration cannot be overcome and that photoinhibition effects prevailed. The chlorophyll content of *Neochloris oleoabundans* grown at 200 µmol $m^{-2}$ $s^{-1}$ is about 1.9 times higher than when cultivated at 500 µmol $m^{-2}$ $s^{-1}$, whereas the carotenoid content was about 1.6 lower, both demonstrating photoacclimation effects. The elevated oxygen concentration in the growth medium as such do not affect the pigment content at sub- and near saturating light conditions. This indicates that elevated oxygen concentrations in the medium do not contribute to more photo oxidative damage at the higher light conditions used, but that oxygen only inhibits the growth via photo respiration effects.

Microalgae grown in closed photobioreactors do not experience constant high concentrations of oxygen. In closed tubular photobioreactors, the oxygen concentrations increase over the tubes due to photosynthesis and drops in the degasser where the surplus of oxygen is removed. In addition, the algae are exposed to the light while residing in the tubes and are exposed to darkness in the degasser. In **Chapter 4**, the dynamically changing oxygen concentrations and subsequent light conditions were simulated in a CSTR at fully controlled conditions at sub-saturating light intensity and the effect on the growth of *Neochloris oleoabundans* was studied at near-saturating light intensity. In addition, the effect of a 10 times elongation of the residence time at in the solar receiver was investigated. In this study, 3 different light regimes were used: continuous light; 30 minutes light on followed by 6 minutes light off and 300 minutes light on followed by 6 minutes light off. The specific growth rate measured at constant low oxygen concentration $P_{O_2}$=0.21 bar during these 3 light regimes were; 1.14 ± 0.06 $day^{-1}$; 0.80 ± 0.16 $day^{-1}$ and 1.09 ± 0.05 $day^{-1}$ respectively. The effect of dynamically changing oxygen concentrations from $P_{O_2}$=0.21 bar to $P_{O_2}$=0.63 bar followed by subsequent degassing to $P_{O_2}$=0.21 bar during the dark period resulted in similar specific growth rates. The decrease of the algae specific growth observed when applying different light regimes, shows that the exposure of the algae cells to dark periods in the degasser has bigger

negative impact than the temporary exposure to accumulating oxygen concentrations in the solar receiver. In **chapter 5** a simulation of the dynamic oxygen concentrations felt by the microalgae in tubular photobioreactors under high light intensities was studied as a follow up of chapter 4. When the algae were exposed to constant oxygen concentration and constant high light the specific grow rate was 1.29 ± 0.08 $day^{-1}$. Using a light regime of 30 minutes of light ON followed by 6 minutes lights OFF and degassing resulted in a specific growth rate of 0.84 ± 0.09 $day^{-1}$, the elongation of the time (lights ON) to 300 minutes resulted in 1.18 ± 0.05 $day^{-1}$. When dynamically changing oxygen concentrations were applied, similar specific growth rates were obtained. These results indicate that the algae do not experience the expected photo-oxidative inhibition caused by high oxygen concentration in combination with high light, as long as the oxygen is removed via regular degassing. The temporary exposure of the algae to the darkness in the degasser has more impact on the productivity as it has under low light.

The removal of oxygen in a degasser not only requires energy but it also reduces the overall productivity, as photosynthesis ceases when the algae reside in the dark zone of the degasser. **Chapter 6** is a general discussion about the implementation of our main findings of this thesis. The effects of reducing the energy input for degassing and mixing on the overall energy balance as well as on the overall production costs were evaluated using an economic model. The model was used to calculate the energy and costs associated to microalgal biomass production in the Netherlands for three different systems at 100 ha scale. The two methods adopted to overcome the negative effect of oxygen in microalgal cultures did result in a decrease in biomass production costs. Moreover, it showed that using our findings a positive energy balance for outdoor production of *Neochloris oleoabundans* in closed photobioreactors can be reached.

# SAMENVATTING

Fototrofe microalgen worden als veelbelovende grondstof voor duurzame biodieselproductie beschouwd, aangezien microalgen zonlicht als lichtbron, $CO_2$ uit rookgassen en nutriënten (P, N) uit afvalstromen kunnen gebruiken. Om de outdoor-microalgenproductie in gesloten fotobioreactoren economisch attractief en duurzaam te maken, moeten de kosten voor het mengen en het ontgassen omlaag worden gebracht en de algehele energiebalans moet positief worden. Het mengen is nodig om het licht effectief en efficiënt te verspreiden en voor het toedienen van koolstofdioxide. Het ontgassen is nodig om het fotosynthetisch geproduceerde zuurstof te verwijderen.

Dit proefschrift richt zich op de effecten van de ophoping van zuurstof op de groei van de vet-ophopende microalgen *Neochloris oleoabundans* bij verschillende lichtintensiteiten. Deze studies laten zien bij welke concentraties zuurstof toxisch wordt voor de algen bij verschillende lichtintensiteiten die de algen gedurende de outdoor-productie ondervinden. Dit geeft aan wanneer de zuurstof verwijderd moet worden uit de fotobioreactor en dus wanneer ontgassen nodig is.

De verschillende niveaus van lichtintensiteiten die in buisvormige outdoor-fotobioreactoren gehaald worden, werden *Neochloris oleoabundans* opgelegd terwijl deze gekweekt werd in een volledig gecontroleerde en goed geroerde tankreactor in turbidostat. Onder continue belichting van 200 µmol $m^2$ $s^{-1}$ (subverzadigde lichtintensiteit) werd bij drie verschillende partiële zuurstofspanning ($P_{O_2}$= 0,24; 0,63; 0,84) een specifieke groeisnelheid van 1,38; 1,36 en 1,06 $dag^{-1}$ gemeten. Bij de partiële zuurstofspanning van 0,84 bar werd de partiële koolstofdioxidespanning ($P_{CO_2}$) verhoogd van 0,007 tot 0,02 bar om te testen of de remmende effecten van de fotorespiratie overwonnen kunnen worden. De toevoeging van koolstofdioxide resulteerde inderdaad in een toename van de specifieke groeisnelheid van 1,06 tot 1,36 $dag^{-1}$. De toename in de specifieke groeisnelheid bevestigt dat fotorespiratie plaatsvond en dat het negatieve effect van fotorespiratie overwonnen kan worden door de $CO_2/O_2$ verhouding in het medium te herstellen. In **hoofdstuk 3** werd het effect van de partiële zuurstofspanning op de groei van *Neochloris oleoabundans* getest onder

bijna-verzadigde lichtintensiteit in een compleet gecontroleerde fotobioreactor. Bij de partiële zuurstofspanningen ($P_{O_2}$= 0,24; 0,42; 0,63; 0,84 bar) die getest werden, was de specifieke groeisnelheid 1,36; 1,16; 0,93 en 0,68 $dag^{-1}$. Een verhoging van de $P_{CO_2}$ van 0,007 tot 0,02 bar bij een $P_{O_2}$ van 0,84 bar bij deze hogere lichtintensiteit had geen positief effect op de totale groei van de algen in tegenstelling tot wat er bij subverzadigde lichtintensiteiten gebeurde. Deze resultaten geven aan dat bij verzadigde lichtintensiteiten het remmende effect van de fotorespiratie niet meer overwonnen kan worden en dat fotoinhibitie-effecten de overhand hebben. Het chlorofylgehalte van *Neochloris oleoabundans* die gekweekt was bij 200 µmol $m^{-2}$ $s^{-1}$, was 1,9 keer hoger dan het gehalte van de cellen die bij 500 µmol $m^{-2}$ $s^{-1}$ waren gekweekt, terwijl het carotenoïdegehalte 1,6 keer lager was; beide zijn uitingen van fotoacclimatisatie-effecten. De verhoogde zuurstofconcentratie in het groeimedium had geen effect op het pigmentgehalte bij sub- and bijna-verzadigde lichtintensiteiten. Dit toont aan dat verhoogde zuurstofconcentraties in het medium geen extra foto-oxidatieve schade bij de hogere lichintensiteit veroorzaken, maar dat zuurstof alleen remmend werkt via fotorespiratie.

Microalgen die in gesloten fotobioreatoren groeien, ondervinden geen constante hoge concentraties van zuurstof. In gesloten buisvormige fotobioreactoren neemt de zuurstofconcentratie geleidelijk toe over de lengte van de buis toe door fotosynthese om in de ontgasser snel af te nemen als het overschot aan zuurstof actief verwijderd wordt. Daarnaast zijn de algen aan het licht blootgesteld terwijl ze in de buisvormige reactor blijven en zijn ze aan complete duisternis blootgesteld in de ontgasser. In **Hoofdstuk 4** werden de dynamisch veranderende zuurstofconcentraties en de daarmee gepaard gaande lichtcondities gesimuleerd in een CSTR onder volledig gecontroleerde condities bij subverzadigde lichtintensiteit. Verder werden de effecten op de groei van *Neochloris oleoabundans* bij bijna-verzadigde lichtintensiteiten bestudeerd.

Daarnaast werd het effect van een verlenging van de verblijfstijd in de zonnecollector met een factor 10 onderzocht. In deze studie werden drie verschillende lichtregimes gebruikt: continu licht, 30 minuten “licht aan” gevolgd door 6 minuten “licht uit” en 300 minuten “licht aan” gevolgd door 6 minuten “licht uit”. De specifieke groeisnelheid werd tijdens de drie verschillende

lichtregimes en bij de constante lage zuurstofconcentratie van $P_{O_2}$=0.21 bar gemeten en was respectievelijk 1,14 ± 0,06 $dag^{-1}$; 0,80 ± 0,16 $dag^{-1}$ en 1,09 ± 0,05 $dag^{-1}$. Het effect van de dynamisch veranderende zuurstofconcentraties van $P_{O_2}$=0,21 bar tot $P_{O_2}$=0,63 bar, gevolgd door het ontgassen tot $P_{O_2}$=0,21 bar tijdens de donkere periode, resulteerde in dezelfde specifieke groeisnelheid. De verlaging van de specifieke groeisnelheid van de algen werd vastgesteld wanneer verschillende licht regimes gebruikt werden. Uit de resultaten bleek dat het tijdelijk blootstellen van de algen aan donkere periodes in de ontgasser een groter negatief effect heeft dan het tijdelijk blootstellen aan oplopende zuurstofconcentraties in de zonnecollector.

In **hoofdstuk 5** werd de simulatie van de dynamische zuurstofconcentraties die ondervonden worden door de microalgen in buisvormige fotobioreactoren onder hoge lichtintensiteit bestudeerd, als vervolg op hoofdstuk 4. Wanneer de algen aan constante zuurstofconcentraties en aan constante hoge lichtintensiteiten blootgesteld waren, was de specifieke groeisnelheid 1,29 ± 0,08 $dag^{-1}$. Wanneer een lichtregime van 30 minuten “licht aan” gevolgd door 6 minuten “licht uit” en ontgassen toegepast werd, resulteerde dit in een specifieke groeisnelheid van 0,84 ± 0,09 $dag^{-1}$, en de verlenging van de tijd (“licht aan”) tot 300 minuten resulteerde in een groeisnelheid van 1,18 ± 0,05 $dag^{-1}$. Wanneer de algen werden blootgesteld aan dynamisch veranderende zuurstofconcentratie, werd dezelfde groeisnelheid gemeten. Deze resultaten tonen aan dat algen de fotooxidatieve inhibitie niet ervaren door hoge zuurstof concentraties in combinatie met hoge lichtintensiteit, zolang de zuurstof regelmatig verwijderd wordt door middel van ontgassen. De tijdelijke blootstelling van de algen aan de duisternis in de ontgasser heeft meer effect op hun productiviteit dan de blootstelling aan lage lichtintensiteiten. Het verwijderen van zuurstof in een ontgasser verbruikt niet alleen energie, maar het reduceert ook nog de totale productiviteit, omdat de fotosynthese ophoudt wanneer de algen in de donkere zone van de ontgasser verblijven.

**Hoofdstuk 6** is een algemene discussie over de implementatie van onze hoofdbevindingen uit dit proefschrift. De effecten van het reduceren van de gebruikte energie voor het ontgassen en mengen op de totale energiebalans en de totale productiekosten werden in een economisch model geëvalueerd. Dit

model werd gebruikt om de energie en de kosten bijbehorend aan de microalgenbiomassaproductie in Nederland voor drie verschillende systemen op een schaal van 100 ha te berekenen. De twee methodes die overgenomen werden om de negatieve effecten van zuurstof in microalgenculturen te overwinnen, resulteerden in een verlaging van de biomassaproductiekosten. Bovendien toonde de analyse aan dat wanneer onze bevindingen gebruikt worden, een positieve energiebalans voor de outdoor-productie van *Neochloris oleoabundans* in gesloten fotobioreactoren bereikt kan worden.

# SUMÁRIO

As microalgas fotótroficas são consideradas uma fonte promissora de matéria-prima para a produção sustentável de biodiesel, devido ao uso da luz solar natural como fonte energética e utilização do $CO_2$ proveniente de gases de combustão e nutrientes (P, N) obtidos pela degradação biológica de resíduos. Para que a produção em larga escala de microalgas em fotobiorreatores seja economicamente viável e sustentável, os custos da mistura e desgaseificação deverão ser minimizados e o balanço energético global deve ser positivo. A mistura é necessária para o fornecimento eficiente de luz e dióxido de carbono, enquanto a desgaseificação é necessária para a remoção de oxigénio gerado fotossinteticamente.

O estudo efectuado centra-se no impacto da acumulação de oxigénio no crescimento da microalga oleaginososa *Neochloris oleoabundans* sob diferentes intensidades de luz. A investigação efectuada demonstrou quais as concentrações de oxigénio que se tornam tóxicas para as algas, nas diferentes condições de luz que podem ser verificadas no cultivo ao ar livre. Foi ainda identificado o timing ideal para a remoção de oxigênio do fotobioreactor, bem como a consequente necessidade de desgaseificação da cultura.

Foram testados diferentes níveis de oxigénio que são atingidos na produção de microalgas *Neochloris oleoabundans* em fotobiorreatores tubulares dispostos ao ar livre, num reactor tanque perfeitamente agitado em condições controladas e regime de turbidostato. Sob iluminação contínua de 200 $m^{-2}$ $s^{-1}$ (intensidade de luz sub-saturante), a taxa específica de crescimento da *Neochloris oleoabundans* foi de 1,38; 1,36 e 1,06 $dia^{-1}$, medida a três diferentes pressões parciais de oxigénio ($P_{O_2}$= 0.24; 0.63 e 0.84 respectivamente). À pressão parcial de oxigénio de 0,84 bar, a pressão parcial de dióxido de carbono ($P_{CO_2}$) foi aumentada de 0,007 para 0,02 bar, visando testar se o efeito inibidor da fotorrespiração poderia ser superado. Verificou-se que a adição de dióxido de carbono resultou num aumento da taxa específica de crescimento, de 1,06 para 1,36 $dia^{-1}$. O aumento da taxa específica de crescimento confirmou que a fotorrespiração inibe o crescimento, e que esse efeito negativo pode ser superado restaurando a relação $CO_2/O_2$.

No **capítulo 3** descreve-se o efeito da pressão parcial de oxigénio no crescimento da microalga *Neochloris oleoabundans* a uma intensidade da luz near-saturante num fotobioreactor em condições controladas. Às pressões parciais de oxigénio testadas ($P_{O_2}$ = 0,24, 0,42, 0,63, 0,84 bar), a taxa específica de crescimento foi de 1,36, 1,16, 0,93 e 0,68 $dia^{-1}$, respectivamente. Um aumento de $P_{CO_2}$ de 0,007 para 0,02 bar sob $P_{O_2}$ 0,84 bar a esta intensidade de luz mais forte, não mostrou qualquer efeito positivo sobre o crescimento global das algas, ao contrário do que acontece em intensidades sub-saturantes. Estes resultados indicam que a intensidades de luz saturantes, o efeito inibitório do oxigênio por fotorrespiração não pode ser superada e que os efeitos da fotoinibição prevalecem. O teor de clorofila de *Neochloris oleoabundans* cultivada sob 200 µmol $m^{-2}$ $s^{-1}$ é cerca de 1,9 vezes maior do que quando cultivada a 500 µmol $m^{-2}$ $s^{-1}$, enquanto que o teor de carotenóides é cerca de 1,6 menor; demonstrando efeitos de fotoaclimatação. A elevada concentração de oxigénio no meio de cultura, por si só, não afecta o teor em pigmentos sob condições de luz sub-saturantes e near-saturantes . Isto indica que concentrações elevadas de oxigénio não contribuem para o aumento de danos foto-oxidativos no meio, nas condições de luz mais elevadas utilizadas, mas que o oxigénio apenas inibe o crescimento por meio de efeitos de foto-respiração.

As microalgas cultivadas em fotobiorreatores não estão expostos a altas concentrações de oxigênio constantes. Em fotobiorreatores tubulares, as concentrações de oxigênio aumentam ao longo dos tubos devido à actividade fotossintética, diminuindo no desgaseificador, onde o excedente de oxigênio é removido. Além disso, as algas estão expostas à luz enquanto no interior dos tubos, e no escuro quando no desgaseificador. No **capítulo 4**, as concentrações de oxigénio dinamicamente variáveis e consequentes condições de luz foram simuladas num CSTR a condições controladas sob intensidade de luz sub-saturante e near- saturante, tendo sido estudado o efeito sobre o crescimento da *Neochloris oleoabundans*. Adicionalmente, foi avaliado o efeito de um alongamento de 10 vezes o tempo de permanência no receptor solar. Neste estudo foram utilizados 3 regimes diferentes: de luz contínua: 30 minutos de luz seguidos de seis minutos de escuro, e 300 minutos de luz seguidos de seis minutos de ausência de luz. A taxa específica de crescimento medida à concentração de oxigénio constante de $P_{O_2}$=0.21 bar durante os 3 regimes de luz

foram respectivamente de 1,14 ± 0,06 $dia^{-1}$; 0,80 ± 0,16 $dia^{-1}$ e 1,09 ± 0,05 $dia^{-1}$. O efeito de alteração dinâmica de concentração de oxigénio, de $P_{O_2}$=0.21 bar para $P_{O_2}$=0.63 bar, foi seguido de subsequente desgaseificação para $P_{O_2}$=0.21 durante o período de ausência de luz, tendo resultado em taxas específicas de crescimento semelhantes. A diminuição da taxa especifica de crescimento observada quando aplicados regimes de luz diferentes demonstra que a exposição das algas a períodos de escuridão no desgaseificador tem maior impacto negativo do que a exposição temporária a altas concentrações de oxigénio no receptor solar. No **capítulo 5** descreve-se o estudo de uma simulação das concentrações de oxigénio dinâmicas sentida pelas microalgas em fotobiorreatores tubulares sob intensidades de luz elevadas. Quando as algas foram expostas a concentração de oxigénio constante e luz elevada constante, a taxa específica de crescimento foi de 1,29 ± 0,08 $dia^{-1}$. Num regime de luz de 30 minutos seguidos de 6 minutos de luz desligada e desgaseificação, a taxa de crescimento observada foi de de 0,84 ± 0,09 $dia^{-1}$. O prolongamento de tempo (luzes acesas) para 300 minutos resultou em 1,18 ± 0,05 $dia^{-1}$. Quando impostas concentrações de oxigênio dinâmicas, foram, obtidas taxas específicas de crescimento semelhantes . Estes resultados indicam que as algas não experimentam a inibição foto-oxidativa esperada, causada pela alta concentração de oxigénio em combinação com a luz elevada, desde que o oxigénio seja removido através de desgasificação regular. A exposição temporária das algas ao escuro no desgaseificador tem mais impacto sobre a produtividade, tal como aconteceu sob intensidades de luz mais baixas.

A remoção de oxigénio no desgaseificador não só requer energia como também reduz a produtividade global, sendo que a fotossíntese cessa quando as algas se encontram na zona escura do desgaseificador. No **capítulo 6** discute-se a implementação das principais conclusões deste estudo. Foram avaliados os efeitos da redução da energia necessária para a desgaseificação e mistura no balanço global de energia, bem como sobre os custos globais de produção, utilizando um modelo económico. O modelo foi usado para calcular a energia e os custos associados à produção de biomassa de microalgas nos Paises Baixos, em três sistemas diferentes, a uma escala de 100 ha. As duas metodologias utilizadas para superar o efeito negativo do oxigénio em culturas de microalgas resultaram numa diminuição dos custos de produção de biomassa. Os resultados alcançados

neste estudo demonstram ser possível atingir um balanço energético positivo na produção de microalgas *Neochloris oleoabundans* em fotobiorreatores dispostos ao ar livre.

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Cláudia.

## ABOUT THE AUTHOR

Cláudia Sousa was born on the 21st of October 1980 in Felgueiras, Portugal. After High school in her hometown, she started studies in biological engineering with the University of Minho, in Braga, Portugal. During her last year, she went to the Netherlands to do her master thesis at Wetsus, making the characterization of an experimental set-up for studying membrane fouling. After getting her MSc. diploma in Portugal, she came back to the Netherlands to start her PhD project in collaboration between the algae theme of Wetsus and the Bioprocess Engineering group of Wageningen University.

# OVERVIEW OF COMPLETED TRAINING ACTIVITIES.

## Discipline specific activities

Advanced Course on Microbial Physiology and Fermentation Technology (2010)
Bioreactor Design and Operation (2010)
Solar Biofuels from Microorganisms (2009)
Wetsus theme meetings (2009, 2010, 2011 & 2012)

## General courses

Working Safely in Laboratories (2011)
PhD scientific writing (2010)
PhD presentation skills (2009)
VLAG PhD week (2009)

## Conferences

1st International Algal Conference, Amsterdam, The Netherlands (2008)
4th Congress of the International Society for Applied Phycology (ISAP), Halifax, Nova Scotia, Canada (2011)
Wetsus congress (2009, 2010, 2011 & 2012)

## Optionals

PhD excursion Spain (2012)
PhD excursion USA (2010)
Wetsus water challenge (2009)
Preparation project proposal (2008)

## Teaching

Bioprocess design - working classes